

**MOLECULAR MECHANISMS INVOLVED IN ARSENIC
PERCEPTION AND TOLERANCE
IN *Arabidopsis thaliana***



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MOLECULAR MECHANISMS INVOLVED IN ARSENIC PERCEPTION AND TOLERANCE IN *Arabidopsis thaliana*

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Dedicated To,



ಏನಾದರೂ ಆಗು ಮೊದಲು ಮಾನವನಾಗು.

Whatever you become, first become a human being.

ಕುವೆಂಪು(Kuvempu)

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ABBREVIATIONS

As: Arsenic

As(V): Arsenate

As(III): Arsenite

CKs: Cytokinins

ROS: Reactive oxygen species

PCs: Phytochelatins

GSH: Glutathione

NIP: nodulin 26-like intrinsic proteins

PSR: Phosphate Starvation Response

UPS : Ubiquitin-proteasome system

TF: Transcription Factor



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SUMMARY

Arsenate is a toxic metalloid to living organisms. The incorporation of this toxic element to soil and water by anthropogenic and volcanic activities pose continuous threat to sessile organisms. Because of close chemical similarity between arsenate [As(V)] and phosphate (Pi), As(V) uses the Pi transporter to enter plant cell, which makes plants vulnerable for this metalloid. Suppression of Pi transporters is a strategy widely used by plants in nature to prevent arsenate uptake. However, closing Pi-transporters compromises phosphate availability and thus plant survival. Currently it is completely unknown, how plants manage this delicate situation preventing arsenic uptake without impairing phosphate acquisition.

Here we found that in response to arsenate, the Pi/As(V) transporter *PHT1;1* rapidly repressed its expression during initial 2 to 8 hrs of As(V) exposure. The early repression of the Pi-transporter is regulated by WRKY6, a repressor that is upregulated by As(V). The repression is coordinated by the degradation of PHR1, a transcriptional activator of *PHT1;1*. The repression is also accompanied by delocalization of the transporter from the plasma membrane. All these responses lead to an instant suppression of As(V) uptake. PHR1 degradation is mediated by ASK18, an SKP1-like protein that is transcriptionally regulated by As(V). Interestingly, ASK18 responsiveness depends on the arsenic detoxification status of plants.

Furthermore, we show that plants reduce their endogenous cytokinin (CK) content as an early response to As(V) that leads to increase detoxification capacity. *PHT1;1* repression and CKs reduction thus allows plants to coordinately reduce As(V) uptake and increased capacity to detoxify intracellular arsenic. Accordingly, transgenic lines with reduced levels of endogenous CKs show remarkable arsenic tolerance phenotype. Surprisingly, after 24 hrs of As(V) exposure *PHT1;1* repression is abolished and the expression is reactivated even in the presence of As(V). Here we found that *PHT1;1* expression recovery is controlled by PHR1 stabilization that is also dependent on plant detoxification capacity. Overall the plants are able to adapt arsenic uptake to their detoxification capacity.

RESUMEN

El arsenato es un metaloide tóxico para los seres vivos. La incorporación de este elemento tóxico al suelo y al agua por actividades antropogénicas y volcánicas supuso una amenaza continua para los organismos sésiles. Debido a la estrecha similitud química del [As(V)] con el fosfato (Pi), el As(V) usa el transportador de Pi para entrar en las células vegetales, lo que hace a las plantas particularmente vulnerables a este metaloide. La supresión de los transportadores de Pi es una estrategia ampliamente utilizada por las plantas en la naturaleza para prevenir la absorción de arsenato. Sin embargo, el cierre de los transportadores de fosfato compromete la disponibilidad de este elemento y, por tanto, la supervivencia de la planta. Actualmente se desconoce el modo en que las plantas gestionan esta delicada situación, previniendo la absorción de arsenato sin dificultar la adquisición de fosfato.

Hemos descubierto que el transportador de As(V)/Pi PHT1;1 reprime rápidamente su expresión en respuesta a arsenato, durante las 2 a 8 horas iniciales de exposición a dicho metaloide. La represión temprana del transportador de Pi es regulada por *WRKY6*, un represor cuya expresión aumenta en respuesta a As(V). Dicha represión está coordinada por la degradación de *PHR1*, un activador transcripcional de *PHT1;1*, y además está acompañada por la deslocalización del transportador de la membrana plasmática. Todas estas respuestas llevan a una supresión inmediata de la absorción de As(V). La degradación de *PHR1* está mediada por *ASK18*, una proteína del tipo *SKP1* cuya transcripción está regulada por As(V). Curiosamente, la respuesta de *ASK18* depende del estado de detoxificación de arsénico de la planta.

Además, hemos demostrado que las plantas reducen su contenido endógeno en citoquininas (CK) como respuesta temprana al As(V) lo cual produce un aumento en la capacidad de detoxificación. Por tanto la represión de *PHT1;1* y la reducción de CKs permite a las plantas dar una respuesta coordinada, reduciendo la absorción de As(V) e incrementando la capacidad de detoxificación del arsénico intracelular. Así, líneas

transgénicas con niveles reducidos de CKs endógenas muestran un fenotipo significativo de tolerancia a arsénico. Sorprendentemente, tras 24 horas de exposición a As(V), la represión de PHT1;1 se anula, reactivando su expresión incluso en presencia de As(V). Hemos encontrado que la recuperación de la expresión de PHT1;1 está controlada por la estabilización de PHR1, la cual también depende de la capacidad de detoxificación de la planta. En resumen, las plantas son capaces de adaptar la absorción de As(V) a su capacidad de detoxificación.

INTRODUCTION

1. Arsenic – A Threat to Life Forms

Arsenic (As) is a toxic metalloid to living organisms that pose serious health problems to mankind such as hyperpigmentation, keratosis, cardiovascular diseases and also it is a potential carcinogen. Arsenic, incorporated to environment by various means of natural and anthropogenic activity is a continuous threat to all organisms. Volcanic eruptions, weathering of rocks and minerals are the natural sources of arsenic contamination; anthropogenic activities like use of arsenic based pesticides, herbicides and release of industrial contaminated water to rivers are increasing arsenic contamination in present days.

Arsenic is a non-essential metalloid for organisms, in fact is highly toxic to plants; it inhibits root growth and development and upon translocation to the aerial part, it blocks overall growth of the plant by compromising fertility, leading to loss of yield and fruit production. Higher concentration of arsenic, interfere with critical metabolic processes leading to plant death. Arsenic enters humans and animals food chain through tainted drinking water or the use of food crops irrigated with As contaminated water. Rice is the major crop grown in the areas like South-east Asia where, As contamination is enormous. Therefore the people who use rice as their staple food are under the threat of As poisoning (Nordstrom, 2002; Meharg et al., 2009). Indeed in Bangladesh, part of India and China is responsible for the worst massive poisoning the humans ever suffered.

1.1 As toxicity mechanisms

Depending upon the redox and pH of soil, As occurs predominantly in two inorganic forms as arsenate [As(V)] and arsenite [As(III)]. The close chemical similarity between As(V) and Phosphate (Pi) enables As(V) to enter to plant cell through Pi transporter (Catarcha et al 2007; Wu et al., 2011). Once inside the plant cell it can move from cell to cell using various Pi transporters which lead to the rapid distribution of As(V) throughout the plant by exposing cellular metabolism to the toxicant (Wysocki and Tamas ; 2010). The

main reason of As(V) toxicity may be the replacement of Pi in critical biochemical reactions (Park and Agrawal, 1972; Long and Ray, 1973; Gresser, 1981). Cellular metabolic pathways such as glycolysis, oxidative phosphorylation, phospholipid metabolisms are extremely As(V)-sensitive which even can also affects DNA and RNA metabolism (Finnegan et al., 2012). In fact, some of the key metabolic enzymes like F_1F_0 -type ATP synthases, or GAPDHases can use As(V) as substrate in their reactions (Gresser, 1981; Orsit and Cleland, 1972). The As(V)-esters produced in these reactions are highly unstable and the enzymatic reactions are thus irreversible (Rosen et al., 2011; Finnegan et al., 2012) causing an uncoupling of the oxidative phosphorylation and photo-phosphorylation pathways which collapses the ATP production in the cell (Watling-Payne and Selwyn, 1974; Wickes and Wiskich, 1975).

The mode of action of As(III) is entirely different from that of As(V). Once inside the plant cell As(V) is readily reduced to As(III) which is highly toxic to plants. Also, As(III) is the predominant form of As in anaerobic soils like flooded rice fields; the existence of As(III) transporters easily incorporates As(III) into plant cells (Zhao et al., 2009). As(III) is a thiol reactive compound which can reacts with very important macromolecules such as the antioxidant glutathione (GSH) or binds to proteins containing thiol groups disrupting their proper folding (Bergquist et al., 2009; Ramdan et al., 2007). Moreover, As(III) causes the disruption of actin and tubulin cytoskeleton (Thorsen et al., 2009). The process of reduction of As(V) to As(III) uses glutathione (GSH) as reductant and leads to the depletion of glutathione. Since GSH is one of the main antioxidant to neutralize reactive oxygen species (ROS), the lack of sufficient GSH causes oxidative stress due to ROS. (Hartley-Whitaker et al., 2001). Glutathione is also a precursor of phytochelatins (PCs), As(III)-chelating-peptides, which are synthesized upon exposure to inorganic arsenic. The synthesis of PCs in turn, results in glutathione depletion, reducing the amount of antioxidant available for quenching ROS, and causing an oxidative stress to the plant cells (Sneller et al., 1999; Hartley-Whitaker et al., 2001; Meharg., 2002).

1.2 Phytoremediation: A promising approach to mitigate As contamination

To overcome the detrimental effect of As there are mainly two strategies: 1) Removal of As from the polluted environment or 2) Development of safe cultivars which can be grown in As contaminated soil but minimizing the uptake and translocation of As to edible part (Tripathi et al., 2007). Many physical and chemical remediation technologies were applied to clean up mechanically the contaminated sites or stabilizing arsenic in the soil but these technologies are expensive and it is not economically viable to apply for large areas (Mondal et al., 2006). The biological method such as phytoremediation is one of the most promising approach which uses collection of plant-based technologies to clean polluted environments (Wu et al 2010; Lasat., 2002). Use of naturally available hyper accumulators such as the fern *Pteris vittata* L., is one promising approach for phytoremediation (Ma et al., 2001). Limitations in adaptability to diverse weather conditions it is not feasible to use this species worldwide. Therefore, it is necessary to develop/engineer ideal plants for efficient phytoremediation with the following characteristics; 1) The ability to hyper accumulate metals, 2) Fast growth and higher biomass production, 3) Adaptable to wide range of weather conditions, 4) Easy to cultivate, 5) with wide and deep root system and 6) No Translocation of metal to edible part (Danh et al., 2014). To develop these genetically engineered highly efficient plants it is mandatory to understand the mechanistic details of As uptake, plant distribution and detoxification process in plants.

2. Molecular basis of arsenic uptake and detoxification in plants.

To alleviate As(V) toxicity, plants developed different strategies such as decreasing arsenic uptake, extrusion of accumulated arsenic, chelation and sequestration to vacuoles and formation of less toxic organic As molecules.

2.1 Arsenic uptake and extrusion mechanisms.

As(V), the analog of phosphate is the predominant form in aerobic soils. In both prokaryotes and eukaryotes As(V) is taken up by using phosphate transporters. Pit and Pst are the two Pi transporters involve in As(V) uptake in *E.coli*. In contrast, the As(III) which structurally mimics glycerol is transported through the aquaglyceroporin GlpF and another

protein ArsB plays role in extrusion of As(III) from the cell. In the legume symbiont *Sinorhizobium meliloti*, it is demonstrated that the aquaglyceroporin AqpS acts as bi-directional channel of As(III) by facilitating uptake and extrusion of As(III) depending on the concentration gradient (Figure A) (Rosen and Tamas., 2010). In budding yeast *Saccharomyces cerevisiae*, similarly to *E.coli*, As(V) is transported through high affinity Pi transporters PHO84 as well as low affinity transporter Pho87 and PHO88. The As(III) enters the yeast cell using aquaglyceroporin Fps1p and hexose transporters. Similar to bacteria, yeast also possesses arsenic efflux mechanisms to reduce the toxic effect of arsenic. Bobrowicz et al., (1997) isolated the *ACR3* gene encoding an As(III) extrusion pump. Yeast cells expressing multiple copies of *ACR3* accumulate less As(III) than the wild type yeast. By the contrary, the deletion of *ACR3* causes sensitivity to As(III) and the yeast exhibits reduced As(III) efflux (Bobrowicz et al., 1997).

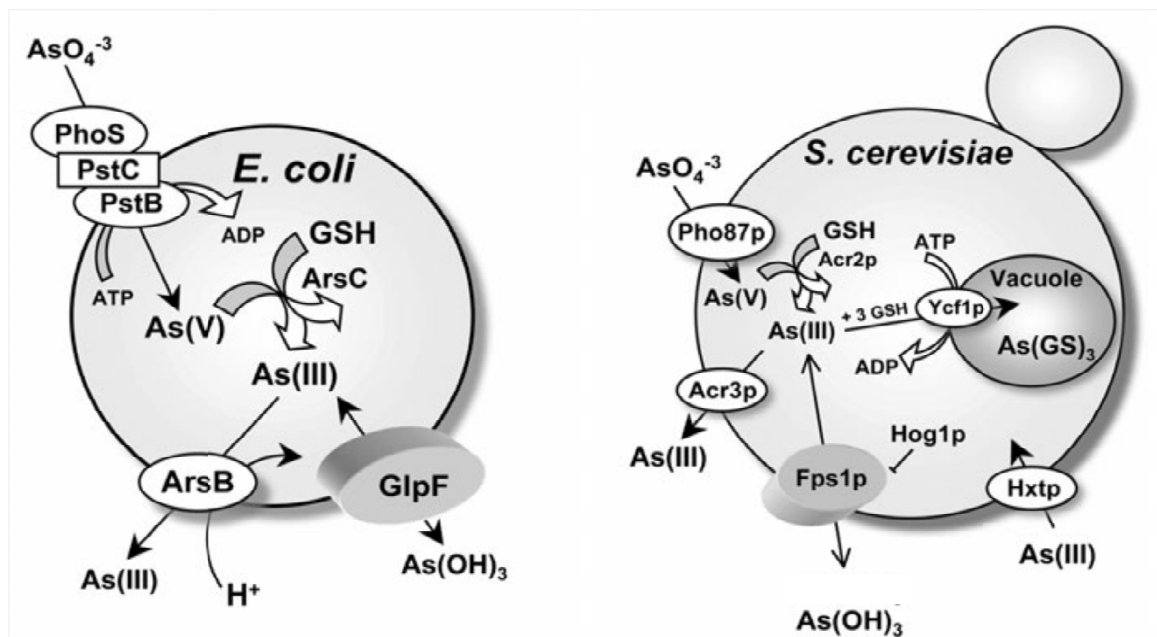


Figure A: Pathways of arsenical uptake and detoxification in *E. coli* and *S. cerevisiae*.

In both *E. coli* and yeast As(V) is taken up by phosphate transporters, while As(III) is taken up by aquaglyceroporins (GlpF in *E. coli* and Fps1p in yeast). In yeast As(III) is also taken up by a number of the hexose transporters (Hxt1p). In both *E. coli* and *S. cerevisiae*, As(V) is reduced to As(III) by an As(V) reductase, in *E. coli* this is ArsC and in yeast it is Acr2p. As(III) detoxification results from removal of the metalloid from the cytosol. In *E. coli*, As(III) is extruded from the cells by ArsB. In yeast, Acr3p is a plasma membrane As(III) efflux protein, and Ycf1p, an ABC transporter in the MRP family, pumps As(GS)_3 into the vacuole (Image from Rosen and Tamás, 2010).

In plants, molecular and physiological studies shown that As(V) and phosphate use the same transporter to enter the cell (Meharg et al., 1994; Catarecha et al., 2007) while As(III) is transported through NIP aquaporins (Zhao et al., 2008). Organic As species such as monomethyl arsenic acid (MMA) and dimethylarsenic acid (DMA) also enters plant cell but the detailed uptake mechanism is currently unknown (Meharg et al., 2004) (Figure B). Reduced level of As(V) uptake by suppressing the As(V)/Pi transporter is the first strategy employed by plants in nature to survive against As. The plant kingdom has more than 100 Pi transporters (PHT1 family), most of them are strongly expressed in roots according to their role in Pi uptake from the soil (Bucher, 2007). In *Arabidopsis* PHT1;1 and PHT1;4 transporters are responsible for Pi uptake and the double knock out mutant *pht1;1/pht1;4* shows significant As(V) tolerant phenotype indicating that As(V) mainly uses these transporters to enter plant cell (Shin et al., 2004). In *Arabidopsis* and rice the mutants defective in PHF1 (PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1), the protein involve in proper localization of Pi transporters in the plasma membrane (Gonzalez et al., 2005; Chen et al., 2011) exhibits remarkable As(V) tolerance which further supports the role of PHT1;1 in As(V) uptake. In our laboratory Catarecha et al (2007) identified a new mutant allele of PHT1;1 in *Arabidopsis* (*pht1;1-3*) which exhibits decreased As(V) uptake but enhanced accumulation of As(V). In the case of *Holcus lanatus* and *Chlamydomonas*, As(V) tolerance is achieved by reducing As(V) uptake through suppression of high-affinity transporters (Bleeker et al., 2003; Murota et al., 2012). These studies indicate that, characterization of the molecular regulation of Pi transporters would give more opportunities to understand As(V) tolerance strategies in plants.

Apart from As(V), plants also incorporate As(III) through their roots. As(III) is the dominant form of As in reducing environments like flooded rice fields being responsible for arsenic contamination of rice grains (Xu et al., 2008). As(III) uptake in plants occurs through nodulin 26-like intrinsic proteins (NIP) which are subfamily of plant aquaporins (Zhao et al., 2010). In rice OsNIP2;1, which function as a silicon transporter, plays a role in As(III) uptake. Expression of this gene in *xenopus laevis* oocytes and in yeast, showed significant uptake of As(III) but not As(V). In addition to OsNIP2;1 other transporters like OsNIP1;1, OsNIP3;1 also take part in As(III) incorporation into the plant cell. While silicon

transporter 1 (Lsi1) transports As(III) into the root cells, the other transporter Lsi2 mediates the efflux of As(III) to the xylem vessels (Ma et al., 2008). Plant species like *A. thaliana*, *Holcus lanatus*, wheat, maize and barley, extrude As(III) to the external media to reduce As toxicity (Zhao et al., 2009). Zhao F.J et al., (2010) reported that Lsi1 is responsible for As(III) efflux thus acting as a bidirectional pump. However, this efflux accounts for only 15–20% of the total As(III) extruded which suggests the existence of other As(III) transporters currently unknown.

2.2. Detoxification of arsenic.

Apart from suppression of As(V)/Pi transporter or As(III) extrusion, detoxification of intracellular As is a major factor adapted by the organisms to cope with the metalloid. Detoxification of As involves two steps 1) The reduction of As(V) to As(III) and 2) Chelation and sequestration of As(III) into the vacuole.

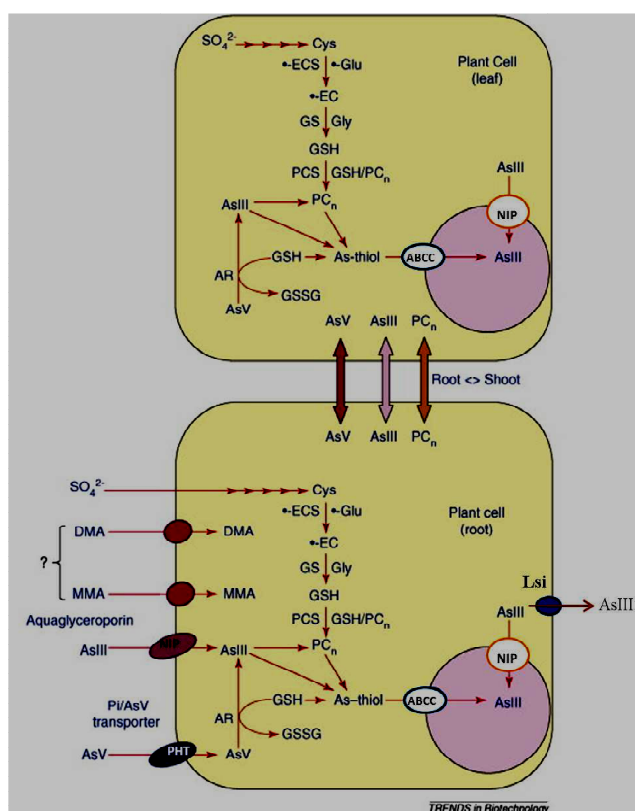


Figure B: Mechanisms of arsenic uptake, translocation and detoxification in plants:

Plants take up As(V) and As(III) through phosphate transporters and aquaglyceroporins respectively. Small amounts of organic As (monomethylarsonic acid, MMA; and dimethylarsinic acid, DMA) can also be taken up through unknown transporters. Long distance transport of As from root-to-shoot takes place in the form of AsV and AsIII. Plants assimilate sulfate to form cysteine (Cys) for the synthesis of glutathione (GSH) in two ATP-dependent steps: in the first, rate-limiting step, γ-glutamylcysteine (gEC) is synthesized by γ-glutamylcysteine synthetase (g-ECS) using cysteine and γ-glutamic acid (γ Glu) as substrates; and in the second step, GSH is synthesized by glutathione synthetase (GS), using glycine (Gly) as a substrate. In response to As, plants induce synthesis of phytochelatins (PCn), the polymers of GSH, through the enzyme phytochelatase (PCS). PCn can be transported from root-to-shoot and vice versa. Before detoxification, As(V) is reduced to As(III) by arsenate reductase (AR) using GSH as a reductant.

Phytochelatins and GSH coordinate with AsIII to form a variety of complexes. These complexes can be sequestered in the vacuole by ABC transporters. In addition, large amounts of unbound AsIII are found in vacuoles, which are transported as free AsIII through NIPs. AsIII effluxed from roots to external media using Lsi transporters. (Modified Figure From Tripathi et al., 2007).

2.2.1 Reduction of Arsenate.

Early in the earth's history, volcanic emissions of As(III) to the biosphere comprised a major threat to incipient life forms (Oremland et al., 2009; Dani, 2010); indeed, all life forms have strategies to cope with this chemical form (Rosen, 2002; Tripathi et al., 2007; Mendoza-Cózatl et al., 2011; Ye et al., 2012). However, when oxygen concentration increased, As(V) suddenly became the most prevalent form of arsenic in the biosphere (Oremland et al., 2009; Dahl et al., 2010; Dani, 2010). Since organisms had not select any As(V) detoxification mechanism, this chemical form was critical for organisms and forced to evolve rapid tolerance responses when As(V) was detected. In fact As(V) is highly toxic since once inside the cell it can substitute most of the Pi dependent reactions. Therefore the ability to reduce As(V) to As(III) through selection of As(V) reductases was the first and most important step adapted by the organisms to overcome arsenic toxicity (Bleeker et al., 2006; Rosen et al., 2002). Indeed, once As(V) enters the cell it is immediately reduced to As(III) by arsenate reductases. In bacteria and yeast three types of cytosolic arsenate reductases were described depending upon the electron donor they use for reduction of As(V). ArsC from *E.coli*, *Staphylococcus aureus* and *Bacillus subtilis*, Acr2p from yeast are the identified arsenate reductases (Mukhopadhyay et al., 2002).

In plants biochemical analysis of arsenic speciation revealed that As(III) is the predominant form present in tissues exposed to As(V). For example in the case of *A.thaliana*, *Brassica juncea*, tomato and rice, As(III) is accounted for 92-99% of the total As (Pickering et al., 2000; Dhankher et al., 2002; Xu et al., 2007) which shows the remarkable efficiency of plants to reduce As(V) to As(III). In cells, As(V) is reduced by both enzymatic and non-enzymatic reactions. Non-enzymatic pathways such as Glutathione (GSH) dependent reduction of As(V) is a relatively slow process which has no biological relevance (Delnomdedieu et al., 1994). In fact, it is described that in *H. lanatus* 90% of As(V) reduction in root protein extract was through enzymatic process using arsenate reductases (Bleeker et al., 2006).

Based on sequence homology with the yeast arsenate reductase Acr2P, the first arsenate reductase (ACR2) was identified in plants from *Arabidopsis*, *H. lanatus*, Rice, and *P.vittata* (Zhao et al., 2009). Expression of ACR2 gene in mutant of *E.coli* lacking arsenate

reductase activity restored the tolerance for As(V). Other heterologous studies confirmed the ability of ACR2 to reduce As(V) (Dhankher et al., 2006) and also it is reported that silencing of *AtACR2* result in As hyperaccumulation in the shoots of *Arabidopsis* (Dhankher et al., 2006). The function of ACR2 is questioned by recent studies (Liu, et al., 2012; Sanchez-Bermejo et al., 2014). Although *AtACR2* is able to complement the arsenate reductase activity in an *E. coli* arsenate reductase mutant, Liu et al (2012) reported that *AtACR2* knockout has no effect on the redox status of arsenic in *Arabidopsis*. In root growth recovery system *Atacr2* T-DNA knockout or *ACR2*-overexpressing lines shows no differences in As(V) sensitivity compared to Col-0. These observations suggest the existence of multiple pathways of arsenate reduction in plants and yeast. Recently, based on natural variation in *Arabidopsis*, Sanchez-Bermejo et al., (2014) identified a new rhodanase protein which showed arsenate reductase activity, thus named *Arabidopsis thaliana* arsenate reductase *QTL1* (AtARQ1). Characterization of this gene shows that *AtARQ1* is transcriptionally inducible by As(V) especially in roots, and the knock-out mutant *Atarq1* is highly sensitive to As(V) but not to As(III), a characteristic of arsenate reductase mutants. Identification of *AtARQ1* demonstrates the existence of a new group of As(V) reductases relevant in As(V) tolerance and detoxification in plants. This role coincides with the enhanced As(V) reductase activity observed in naturally selected As(V) hyper-accumulator plants, which triggers an increase in plant capacity to detoxify As(III) (Sanchez-Bermejo et al., 2014).

2.2.2 Chelation and sequestration of As(III).

The reduced form of arsenic, As(III), is highly toxic. To cope with this situation organisms possess different strategies to neutralize the toxicity of As(III). In *E. coli*, extrusion of As(III) from the cytosol through ArsB transporter is the only mechanisms described to cope with As(III) in bacteria. However, in yeast, in addition to the extrusion pump ACR3p, a vacuole transporter named Ycf1p is present. This protein is an ATP-binding cassette (ABC) transporter homologous similar to the multidrug-associated protein MRP1 that is involve in the sequestration of As(III)-(GS)₃ complex into the vacuoles (Li ZS et al., 1996).

To deal with As(III), plants produced antioxidants and low molecular weight chelators called non protein thiols (NPTs). These peptides are responsible for the heavy metal chelation and its posterior transport into vacuoles. NPTs are small peptides with sulfur containing ligands that includes Glutathione (GSH) and phytochelatins (PCs) (Schmöger et al., 2000). Phytochelatins (PCs) are thiol (SH)-rich peptides with common structure $[\gamma\text{-glu-cys}]_n\text{gly}$ where $n=2$ to 11. PCs are synthesized from GSH through different enzymatic reactions catalyzed by the enzymes γ EC synthetase (GSH1), GSH synthetase (GSH2) and phytochelatin synthase (PCS1). These enzymes are induced in response to As stress (Sung et al., 2009) (Figure C). As(III) reacts, in the cytoplasm, with the thiol groups present in the PCs structure and the resulted compounds can be sequestered into vacuoles using the vacuolar PC transporters, ABCC1 and ABCC2 (Figure B). The lack of these transporters make *A. thaliana* hypersensitive to arsenic (Song et al., 2010). This process of As(III) complexation to PCs not only detoxify As(III) but also prevent its translocation from roots to shoots (Liu et al., 2010). However, there are evidences that, the As hyperaccumulator *Pteris vittata*, is able to store uncomplexed As(III) in the vacuoles (Indriolo et al., 2010). Today, although recent efforts have been made to understand the systems underlying As(V) tolerance (Sung et al., 2009; Song et al., 2010; Mendoza-Cózatl et al., 2011; Sanchez-Bermejo et al., 2014), the molecular mechanisms involved in plant As(V) perception are completely unknown. Indeed, no regulatory genes or transcriptional activators of the As(V) response has been identified so far.

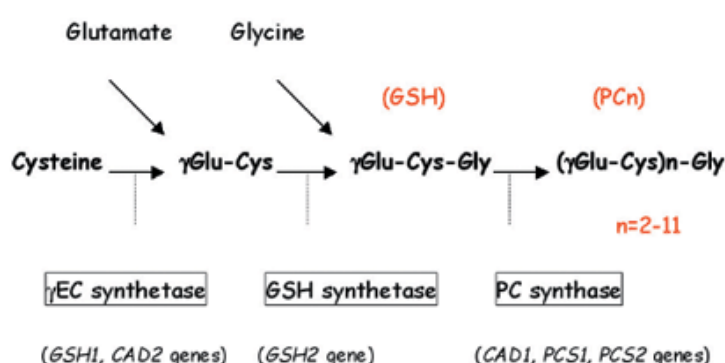


Figure C: Biosynthesis of phytochelatins in higher plants (Image form, Inoue., 2005)

3. Molecular mechanisms involved in arsenic response.

Arsenic adversely affects plant growth and metabolism, causing a reduction in crop yield. To reduce arsenic intake requires better understanding of molecular mechanisms involved in arsenic tolerance to make the crops to withstand arsenic stress. Currently only few molecular mechanisms involved in arsenic tolerance has been dissected but the signaling pathways that sense arsenic or distinguish between As(V) and Pi remain unknown. The studies of Catarecha et al (2007) identified that Pi transporter PHT1;1 is downregulated by As(V). Microarray studies demonstrated that As(V) rapidly repress genes induced by Pi starvation and coordinately induces As(V)-responsive genes that also alters various biological process related to heat and oxidative stresses, phytohormones homeostasis, cell wall architecture and nutrient transport system (Abercrombie JM et al., 2008; Fu SF et al., 2014). The repression of *PHT1;1* by As(V), suggests that As(V) perception, might share components with those operating in the Pi-starvation response. Therefore to dissect the regulation of As perception mechanism, it is important to understand the mechanisms underlying Pi signaling in plants.

3.1. Regulation of the Pi-starvation response

In *Arabidopsis*, in response to Pi deprivation, the high affinity Pi transporter PHT1;1 is up-regulated by the transcription factor PHR1. PHR1 is a central regulator of Pi starvation response; it binds to the P1BS motif present in the promoter region of several Pi starvation-induced genes including the Pi transporters. The mutant *phr1* is impaired in several Pi starvation responses such as: anthocyanin accumulation, root to shoot growth ratio and the expression of some genes that respond Pi starvation (Bustos et al., 2010; Rubio et al., 2001). In turn, PHR1 is posttranslationally regulated by sumoylation (Miura et al., 2005). Recent studies in rice demonstrated that an SPX family protein i.e SPX4, is involved in Pi starvation signalling, acting as a negative regulator of PHR2 the rice ortholog of PHR1. During Pi sufficient condition SPX4 binds to PHR2 and retains it in cytoplasm thereby inhibits the PHR2 from binding to its target genes. During Pi deficiency SPX4 undergo degradation through proteasome pathway and release PHR2 to nucleus to activate phosphate starvation response (Lv et al., 2014). Similarly, a negative regulatory effect on

PHR1 is reported in *A. thaliana* wherein SPX1, which is induced by PHR1 under Pi starvation conditions, in turn exerts negative feedback regulatory loop on PHR1 during Pi sufficiency conditions (Puga et al., 2014). The role and regulation of PHR1 transcription factor during arsenic response remains unknown. PHT1;1 is also regulated at posttranslational level by PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1), a protein located in the endoplasmic reticulum. This protein is necessary for the correct localization of PHT1;1 in the plasma membrane (Chen et al., 2011; González et al., 2005). Another way of regulation involve the phosphorylation of Ser residues located in the C'-end of PHT1;1. When the internal cell level of Pi is high, the phosphorylation of PHT1;1 prevent its exit from the endoplasmic reticulum. Increased Pi concentration delocalize PHT1;1 from the plasma membrane, which leads to the internalization of PHT1;1 in the vacuole by endocytosis and posterior degradation (Bayle et al., 2011). Similarly in *S. cerevisiae* phosphate uptake is dependent on Pi transporter Pho84, and during Pi sufficient condition the Pho84-mediated activation of the protein kinase A (PKA) pathway causes rapid internalization and vacuolar breakdown of Pho84.

PHO2 (UBC24), is an E2 ubiquitin conjugase, a negative regulator of Pi starvation responses, mutant of *pho2* accumulate excess Pi in shoots leading to Pi toxicity accompanied by leaf senescence. Another protein PHO1, load Pi to xylem to transport Pi to shoots. During Pi sufficient condition PHO2 promotes the degradation of PHO1, thereby inhibiting Pi transport from root to shoot and maintains Pi homeostasis (Rojas-triana et al., 2013). In addition to PHO2, another vital component of Ubiquitination pathway controlling Pi homeostasis in plants is NLA (Nitrogen Limitation Adaptation). NLA is an E3 ubiquitin ligase that plays a key role in adapting plants to nitrogen starvation conditions. Recent studies have shown that the *nla* loss of function mutant hyper accumulates Pi, phenocopying the Pi signaling mutant *pho2* under low nitrogen and high Pi conditions (Surya K et al., 2011). While many aspects of the regulation of PHT1;1 during phosphate starvation are known there is no information available regarding the regulation or subcellular localization of this transporter in response to As(V).

Together with PHR1, other transcription factors, members of the WRKY family have been involved in the regulation of the Pi starvation response (Wang et al., 2014; Devaiah et

al., 2007). Interestingly, genome wide transcriptomic analysis in rice and *Arabidopsis* revealed that many transcription factors are differentially regulated in response to arsenic. Among them members of the WRKY family are significantly up regulated. The possible role of these transcription factors in the regulation of the genes related with arsenic response is currently unknown (Abercrombie et al., 2008; Yu et al., 2012; Fu et al., 2014).

3.2 Role of WRKYs in the Pi starvation and other stress responses.

WRKY transcription factors are one of the largest transcriptional regulators families in plants. WRKYs are involved in many processes such as germination, senescence, biotic and abiotic stresses. WRKY proteins have the ability to self and cross regulate their expression by binding to their own promoters and others from the WRKY family. The defining feature of WRKY TFs is their DNA binding domain, which contains sixty amino acid region defined by an N-terminal conserved WRKYGQK motif (Rushton et al., 2010). WRKY TFs regulates their target genes by binding to specific conserved region TTGACC/T called W Box (Ulker and Somssich., 2010). There are a few exceptions where WRKY proteins bind to non-W box sequences. In rice the WRKY13 bind to the pathogen responsive element, PRE4 (TGCGCTT) as well as to W boxes (Cai et al., 2008), whereas in *Nicotiana tabacum* *NtWRKY12* binds specifically to the WK box (TTTTCCAC) (Van Verk, et al., 2008). In *Arabidopsis*, *AtWRKY25* positively regulates the salt and heat stress (Li et al., 2009). In rice, *OsWRKY24* and *OsWRKY45* act as repressors of ABA-inducible promoters while *OsWRKY72* and *OsWRKY77* acts as activators of the same promoter targets (Xie et al., 2005). The over expression of a soybean TF *GmWRKY54* in *Arabidopsis* resulted in more salt and drought tolerant, whereas the over expression of another TF like *GmWRKY13*, resulted in hypersensitivity to salt stress (Zhou et al 2008). Therefore, WRKY proteins act as both transcriptional repressor and activators. For example, in *Arabidopsis* *AtWRKY6* negatively auto regulates its own promoter while activates *SIRK*, which encodes a receptor-like protein kinase, which is strongly induced specifically during leaf senescence (Robatzek, and Somssich 2002). The promoters of many phosphate starvation responsive genes contains one or more W-boxes; for example *Arabidopsis* Pi-responsive genes *At4* that encodes a riboregulator and *PHT1;5* encoding a Pi transporter are both regulated by

WRKY75 (Devaiah, et al 2007; Nagarajan, 2011). *PHO1* responsible for xylem loading of Pi is transcriptionally regulated by WRKY6 and WRKY42. During Pi starvation these TFs bind to the *PHO1* promoter and repress the transcription thereby impairing the Pi transport from roots to shoots (Chen et al., 2009). All these examples support the role of WRKY TFs in transcriptional reprogramming and signaling of diverse biological processes in plants including Pi-response but the role of WRKYs in response to arsenic stress is currently unknown.

4. The role of ubiquitin proteasome system in abiotic stress signaling

In response to environmental stimuli plants activates a cascade of signal transduction pathways that alter gene expression to reshape physiological and cellular processes to overcome the adverse effect of stresses. Stress response in plants is regulated at transcriptional and postranscriptional level. In fact, transcription factors undergo post translational modifications such as farnesylation, phosphorylation, sumoylation or ubiquitination (Lyzenga WJ et al., 2011). Microarray studies of *Arabidopsis* exposed to arsenic showed genes related to the proteasome pathways are upregulated, which indicate the potential role of protein ubiquitination in the degradation of damaged proteins by general stress response e. g. oxidative stress caused by arsenic (Fu et al., 2014). However, it has been propose that protein degradation may play an important role in the regulation of signaling and thus crucial for stimuli perception (Stone 2014). It is repeatedly proved that ubiquitination plays a major role in post-translational modification of regulatory proteins during biotic and abiotic stress responses. Indeed, the ubiquitin-proteasome system (UPS) is an efficient pathway that regulates hormone biosynthesis and other signaling proteins like transcription factors (Stone 2014).

Ubiquitination is a multistep process, where the target protein is ubiquitinated and targeted for degradation using sequential action of three different enzymes such as: Ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) (Figure D). The ubiquitinated substrate protein will be directed for degradation through 26S proteasome, a large multisubunit protease that consists of 20S catalytic core and 19S regulatory subunit. The final step of substrate recognition is dependent on ubiquitin ligase i.e E3 ligase. Different classes of E3 ligases are known (Figure E), one of the

largest and versatile classes is SCF (SKP1-Cul1/Cdc53-F-Box) ubiquitin ligase (Harrison et al., 2005). SCF E3 ligase is a multiprotein complex consist of at least 3 components, Skp1, cullin-1/Cdc53 and Rbx1/Roc1/Hrt1. In addition to this core complex it also contains another variable receptor protein called F-Box protein involved in binding the tagged protein (Lechner et al 2006).

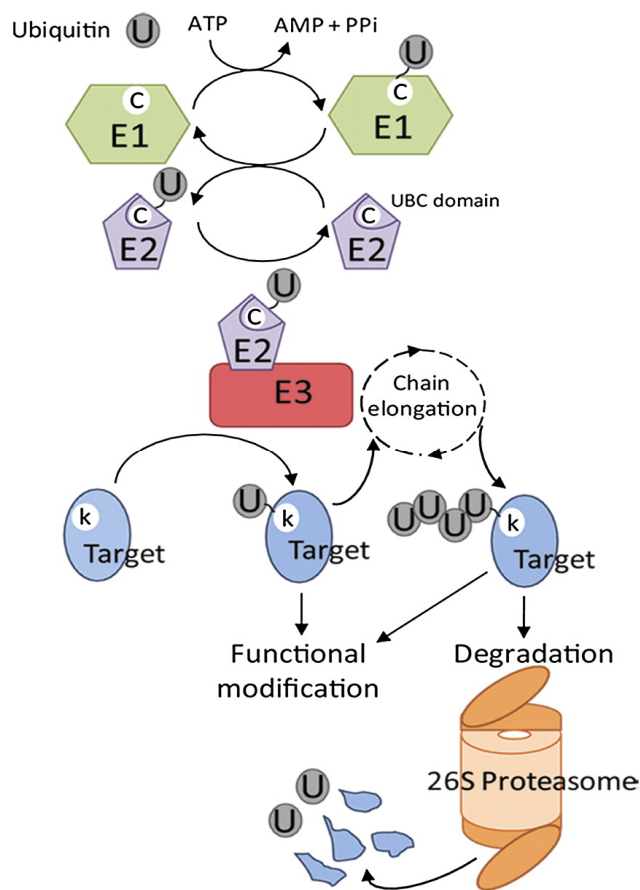


Figure D: The ubiquitination pathway. Ubiquitin is activated by the E1 and then transferred to a conserved cysteine residue on the E2 forming an E2-ubiquitin intermediate. Ubiquitin is transferred from the E2-ubiquitin intermediate to an internal lysine of a target protein bound to the E3 (mono-ubiquitination). Additional ubiquitin molecules can be added to the mono-ubiquitinated target (polyubiquitination) (Image from Lyzenga and Stone, 2012).

Recent advances in the area of the ubiquitin proteasome system (UPS) and abiotic stress provides great opportunity to exploit this mechanism to improve plant tolerance to arsenic. Manipulating the components of UPS, especially the substrate-recruiting E3 ubiquitin ligases, can enhance plants stress tolerance. Several studies demonstrated the successful use of this approach in abiotic stress (Stone 2014). In *Arabidopsis*,

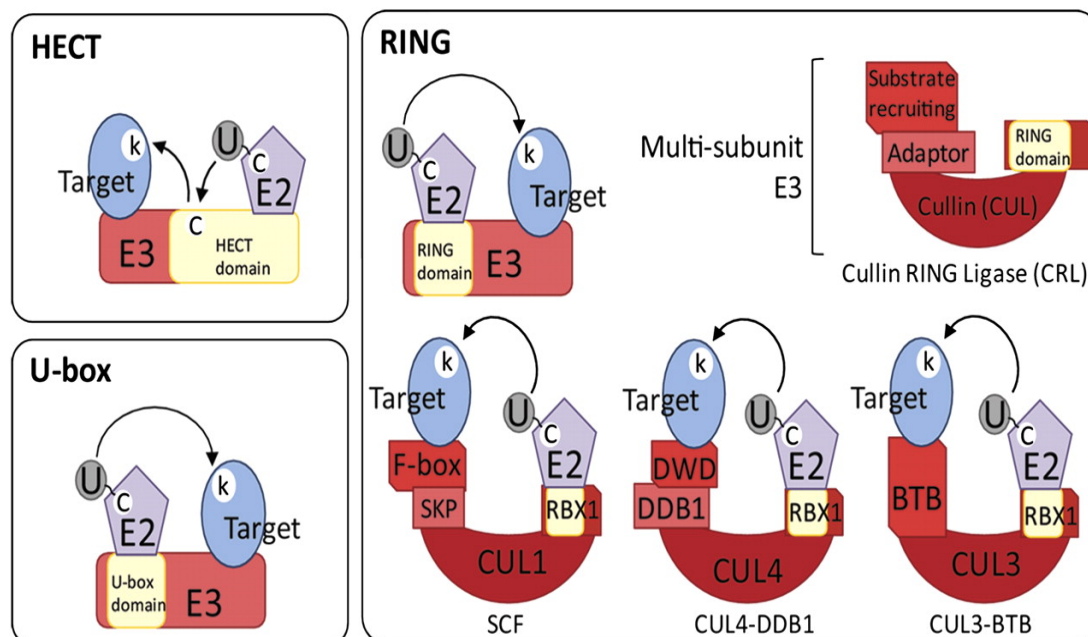


Figure E. E3 ubiquitin ligases. E3s are categorized based on the presence of a RING, HECT or U-box E2 binding domain. The RING-type E3s are subdivided into groups depending on whether the E2 and substrate-binding functions are found in the same protein (monomeric E3s) or on different proteins (multi-subunit E3s; CRLs). The multi-subunit CRL uses a CUL protein as a scaffold to interact with the E2 binding RING protein and a substrate-recruiting protein. The CUL3-based CRLs utilize the BTB substrate-recruiting proteins. CUL1- and CUL4-based CRLs use SKP and DDB1 adaptor proteins, respectively, to bind the F-box and DWD substrate-recruiting proteins (Image from Lyzenga and Stone, 2012).

overexpression of *SDIR1*, a RING-type E3 ligase, which is a positive regulator of ABA signaling, enhanced drought tolerance (Zhanget al., 2007). During drought situations DRIP1 and DRIP2 RING-type E3 ligases negatively regulates their target protein DREB2A to alleviate the effects of stress. Down regulation of DRIP1 and DRIP2 or over expression of stable form of DREB2A improved the drought tolerance in *Arabidopsis* (Qin et al., 2008). Emerging evidence suggest the potential role of UPS pathway in arsenic stress. In *Saccharomyces cerevisiae* the transcription factor Yap8p confers arsenic tolerance by enhancing the transcription of ACR2 and ACR3 genes involved in arsenic detoxification. During arsenic stress Yap8p is degraded through UPS, which depends on the ubiquitin-conjugating enzyme Ubc4p (Di et al., 2007). In yeast during cadmium/arsenic stress the transcription factor Met4 is activated in order to induce glutathione biosynthesis as well as genes involved in sulfur-amino acid biosynthesis. It is described that cadmium and arsenic prevent the Met4 ubiquitination and leads to activation of Met4 through the ubiquitin

ligase SCF^{Met30} (James et al., 2005). In fission yeast, microarray studies revealed that cadmium-inducible genes are under the control of Zip1 transcription factor. Harrison et al (2005) reported that Zip1 arrest the growth of cell during cadmium response to maintain cell viability. Normally growing cell prevents this response by constitutively ubiquitinating and degradation of Zip1 through F-box protein SCF^{pof1}. Therefore, the role of ubiquitin-proteasome pathway as a potential mode of regulation of the arsenic response deserves to be explored in plants.

It is predicted that *Arabidopsis* genome encodes for two E1 enzyme, 37 E2 enzyme and nearly 1300 E3s or components of E3 complexes (Hua and Vierstra., 2011) which suggest that large number of cellular processes are regulated through protein ubiquitination. The microarray studies in *Arabidopsis* plants exposed to arsenic revealed elevated expression of genes related to proteasome pathway which indicates that during arsenic stress the oxidized or damaged protein in the cells might be targeted to ubiquitination for proteasomal degradation (Fu et al., 2014). But the lack of information about the substrate identity of the UPS and their role in plant signaling, limits the understanding of functionality of these induced ubiquitin ligases. Therefore, it is important to identify target proteins/TFs for these ligases, which will open a new approach to further understand the role of the UPS is arsenic stress tolerance.

5. Role of phytohormones in plant adaptation to environmental stress.

Phytohormones are considered as master regulators of plant growth and development, regulates various biological processes like cell division and response to various environmental stresses via complex network of signaling pathways (Kieber et al., 2010). Among several strategies, manipulation of phytohormones is one of the promising alternatives towards obtaining enhanced stress tolerance. However manipulation of these hormones may compromise normal plant growth and development, so tight spatio-temporal regulation of hormones is required in order to minimize possible negative effects (Cabello et al., 2014). Several studies demonstrated the cross-talk between heavy metals and phytohormones. In rice, upon As(III) treatment, genes related to jasmonic acid (JA) pathways are considerably upregulated which indicates the putative role of this hormone

in As(III) stress. Furthermore, other genes encoding proteins involved in ethylene, ABA and cytokinin synthesis were also activated (Yu et al., 2012). Likewise, in the microarray of *Arabidopsis* plants exposed to As(V), transcripts related to ABA, ethylene, brassinosteroid, and cytokinin are differentially regulated. Particularly relevant are downregulation of genes involved in cytokinins (CKs) homeostasis (Fu et al., 2014) because CKs are known for negative regulators of Pi starvation responsive genes including As(V)/Pi transporter *PHT1;1* (Franco-Zorrilla et al., 2002).

CKs are tightly involved in cell growth; division and elongation, during plant development. However cell growth arrest and reactivation are also classical responses of plant adaptation to stress conditions. Stresses like drought and salinity causes the reduction of endogenous CK content and also affected its distribution through the plant (Nishiyama et al., 2011), which indicates the important role of cytokinin in plant adaptation to environmental cues. Cytokinins promote strong cell division, which is a highly energy demanding process. It is reported that short term/mild stress causes the transient elevation of CK levels (Havlova et al., 2008), but when plants were exposed to severe/prolonged stress there is down-regulation of CK content which in turn cause cell growth arrest to reallocate the limited energy resources to cope with the stress situation (Nishiyama et al., 2011).

In response to various abiotic stresses CK metabolic and signaling genes are altered. Several studies identified both positive and negative regulation of different components of cytokinins signaling during stress (Table A) (Ha et al., 2011; Jeon et al., 2010; Nishiyama et al., 2011; Werner et al., 2010; Tran et al., 2007; Mason et al., 2010; and Wohlbach et al., 2008). Studies using plants with reduced endogenous cytokinin levels by over expression of CKX (cytokinin dehydrogenase) protein which degrades CKs or by knockout of CKs biosynthetic genes (IPTs) results in enhanced tolerance to drought and salt stress (Nishiyama et al., 2011; Werner et al., 2010). These results indicate that CKs are negative regulators of stress responses. It is proposed that improved tolerance to drought in CKX overexpressing plants or in knock-out mutants of IPTs is due to increased root biomass and improved cell membrane integrity during water stress in these plants (Nishiyama et al., 2011; Werner et al., 2010). However the molecular basis that promotes stress tolerance in

plants with low CKs endogenous levels are currently unknown and it is conceivable to speculate that reduction of CKs content is one key rescue system for plants adaptation to stress situations.

Gene ID	Description	Regulatory function in stress response	Type of stresses	Regulatory function in ABA response
<i>AHK2</i>	CK receptor kinase	Negative	Cold, drought, salt	Negative
<i>AHK3</i>	CK receptor kinase	Negative	Cold, drought, salt	Negative
<i>AHK4</i>	CK receptor kinase	Negative regulator with CKs	Salt	Negative
<i>CKXs</i>	CK degradation	Positive	Drought, salt	Positive
<i>IPTs^a</i>	CK biosynthesis	Negative	Drought, salt	Negative
Type-B ARR				
<i>ARR1, ARR12</i>	Transcription factors	Negative	Salt	Unknown
Type-A ARR				
<i>ARR3, ARR4, ARR5, ARR6, ARR8, ARR9</i>	Primary response genes	Positive	Salt	Positive
<i>ARR4, ARR7</i>	Primary response genes	Negative	Cold, drought, osmotic	Negative
<i>ARR3, ARR4</i>	Primary response genes	Positive	Cold, drought, osmotic	Positive
<i>ARR8, ARR9</i>	Primary response genes	Negative	Osmotic	Negative
<i>ARR5</i>	Primary response genes	Negative	Cold	None
<i>ARR6</i>	Primary response genes	Negative	Cold	Negative
<i>ARR7</i>	Primary response genes	Negative	Cold	Negative

Table A: Summary of CK metabolic and CK signaling genes involved in abiotic stress response based on published literature (Table from Ha et al., 2012).

Apart from the changes in CK content, CK signaling mechanisms also tightly regulate stress responses. Among the four *Arabidopsis* cytokinin receptors AHK1, AHK2, AHK3 and AHK4/CRE1, the AHK1 positively regulates osmotic stress acting as an osmosensor (Urao et al., 1999; Tran et al., 2007). In contrast, other CK receptors AHK2, AHK3 and AHK4 are negative regulators of stress signaling (Tran et al., 2007). Other components of CK signaling like *Arabidopsis* response regulators (ARRs) have been involved in plant adaptation to low temperatures (Jeon et al., 2010). Mason et al. (2010) identified that ARR1 and ARR12 acts as negative regulators during salt stress response. Although several studies indicate that CK content and signaling pathways negatively regulates stress response in plants, the role of cytokinins during arsenic stress needs further investigation. In this thesis we aim to contribute to the characterization of molecular mechanisms involved in arsenic perception and tolerance in plants using molecular, *in silico* and genetic approaches.

OBJECTIVES

Until date several studies on arsenic response indicates that a crucial strategy of plants to cope with arsenic stress is to reduce As(V) uptake by down regulation of the As(V)/Pi transporter. However, there is no information available regarding the molecular mechanism implicated on the regulation of the transporter during As(V) stress. It is known that the Pi transporters are negatively regulated by cytokinins (Franco-Zorrilla et al., 2002). However, no reports described the importance of cytokinin content during arsenic stress. To bridge this gap, in this thesis, we have undertaken investigation with the following objectives:

- To study the regulation of *PHT1;1* in response to As(V)
- To determine the transcriptome profile in a short-term response to As (V) in *A. thaliana* to uncover new molecular components of arsenic perception.
- To determine the role of PHR1 and other transcription factors in the regulation of the As(V)/Pi transporters during the short-term arsenic response.
- To study the role of cytokinin in Arsenic tolerance.

MATERIALS AND METHODS

1. Plant Materials and Growth Conditions

We used the T-DNA mutant of gene WRKY6 SALK_012997C that bears an insertion in the third exon, as well as several *Arabidopsis thaliana* transgenic lines, including 35S:PHT1;1-GFP (González et al., 2005), CCA1-LUC, CAB-LUC (a kind gift of Salomé Prat [Centro Nacional de Biotecnología–Consejo Superior de Investigaciones Científicas]), PIP1;4-YFP (wave 138) (Geldner et al., 2009) and 35S:HA:GR:PHR1 (Bustos et al., 2010), *cad1-3* (Howden et al., 1995), *pad2-1* (Dubreuil-Maurizi et al., 2011), 35S:CKX1 (Werner et al., 2003).

Seedlings were grown on Johnson medium supplemented with distinct Pi (KH₂PO₄) concentrations depending on the experiment; 1 mM Pi was used for complete medium (+Pi), 30 µM for low-Pi medium, and 5 µM for phosphate starvation (-Pi). In general, for treatment with As(V) (NaH₂AsO₄·7H₂O), plants were grown on Johnson medium +Pi for 7 d, then transferred to -Pi medium for 2 d (supplemented with 5 µM DEX when specified), and finally to liquid -Pi medium alone or supplemented with 30 µM As(V). For all molecular and biochemical analyses, *Arabidopsis* plants were grown in a culture chamber in a 16-h-light/8-h-dark regime (24°C/21°C).

2. Physiological Measurements

For As(V) tolerance analysis, plants were grown on Johnson medium with 15 µM Pi, alone or with 15 µM As(V), in a horizontal position for 10 d. Main root length was then measured using ImageJ software (Barboriak et al., 2005). For arsenic accumulation experiments, plants were grown on plates containing half-strength Bates and Lynch medium solidified with 0.4% bacto-agar with 30 µM Pi and covered with 0.4-mm-pore nylon mesh. Seeds were sown onto the mesh and cultured for 7 d. Plants were treated with PI and PII buffers (Narang et al., 2000). Arsenic accumulation experiments were performed in 50-mL pots using 5 µM As(V). Plants were dried (60°C, 5 d), mineralized with HNO₃-H₂O₂ in a pressure digester, and analyzed for total arsenic content by inductively coupled

plasma–mass spectrometry at the Servicio Inter departamental de Investigación (Universidad Autónoma de Madrid, Spain).

2.1. Quantification of endogenous Cytokinins.

For the quantification of endogenous cytokinins, plants were exposed to 5 μ MAs(V) for 24 h as described and fresh samples were collected and frozen in liquid nitrogen. 250 mg of frozen samples were ground in a mortar with liquid N₂ and homogenized with 4 ml of pre-cooled (–20°C) methanol:water:formic acid (15:4:1, v/v/v). Deuterium-labelled cytokinin internal standards ([²H₅]Z, [²H₅]tZR, [²H₆]iP, [²H₆]iPR, [²H₇] BA, [²H₇]BAR, and [¹³C₅]oT; all from Olchemim Ltd, Olomouc, Czech Republic), were added (40 μ l of a stock solution of 50 ng/ml of each standard in methanol) to the extraction medium and extraction was performed as described in Sudre et al (2013). The extract was injected to HPLC-ESI-MS/MS and cytokinin content was quantified at CIPAV, Timac Agro Espana, Orcoyen, Spain.

2.2. Biothiols quantification

An alkaline-reducing procedure was used to extract bio-thiols from *Arabidopsis* seedlings exposed to As(V), using procedure described by Jocelyn (1987) with minor modifications. 0.1 mg of frozen samples were grinded with 30 μ L of 50 μ M N-acetyl cysteine (N-AcCys) internal standard, and was homogenised with 270 μ L of the reducing reagent (2 mg ml⁻¹ NaBH₄ in 0.1 M NaOH). The mixture was transferred to 1.5 mL Eppendorf tubes, and it was acidified with 50 μ L 10 M HCl, centrifuged at 12,000 x g for 15 min at 4°C, the supernatant was transferred to chromatographic vials kept under dim light. The biothiol profile was analysed by HPLC following the procedure described by Sobrino-Plata et al. (2009). Extracts (100 μ L) were injected in a Mediterranea SEA18 column (250 x 4.6 mm; Teknokroma, Sant Cugat del Vallés, Spain), using an Agilent Technologies 1200 series HPLC system (Santa Clara CA, USA). Thiols were detected after post-column derivatisation with Ellman reagent and quantified against the N-AcCys internal standard. The identity of biothiols was achieved by comparing the retention times of peaks from each sample with those of commercially available standards: Cys and GSH were purchased from Sigma-Aldrich (St. Louis, MO, USA) and PC₂, PC₃ and PC₄ from AnaSpec (Fremont, CA, USA)

3. Binary Construct and Plant Transformation.

To generate the WRKY6-overexpressing lines, full-size WRKY6 cDNA was amplified with primers Wrky6-GWF and Wrky6-GWR, cloned into the pDNR207 vector, and inserted into the pGWB5 binary vector containing the constitutive 35S promoter, using the LR recombination reaction (Invitrogen). This binary construct was introduced into *Agrobacterium tumefaciens* strain C58C1 and transformed in wild-type Columbia-0 (Col-0) plants using the floral dip transformation method (Clough and Bent, 1998). Transformants were selected on 40 mg/L hygromycin containing medium. Homozygous lines were selected for strong expression. Similarly 35S:ASK18/35S:HA:GR:PHR1 double transgenic was developed by cloning ASK18 full length cDNA using ASK18F and ASK18R primers (Table B) into pGWB402 binary vector and transformed into 35S:HA:GR:PHR1 transgenic lines. Transformants were selected on 50mg/L kanamycin and 10mg/L BASTA containing medium.

Approximately 2 kb of the PHT1;1 promoter regions was amplified using primer pairs, PHT1;1p-I/II-F, PHT1;1p-I/II-R. The products were excised with HindIII and BamHI and cloned into the pLUC vector (Ulm et al., 2004). Using these constructs as templates, three versions of the PHT1;1 promoters were generated by PCR site-directed mutagenesis to alter the sequence of the ARE and W-box, respectively; primers used are listed in Table B. All binary constructs were introduced into *Agrobacterium* strain C58C1. *Agrobacterium*-mediated transient expression assays in *Nicotiana benthamiana* plants were performed as described (Jiménez et al., 2006). The PHT1;1 binary constructs were transformed in wild-type Col-0 plants by floral dip transformation. Transformants were selected on 50 mg/L kanamycin-containing medium. Homozygous lines were selected for strong expression.

For bimolecular fluorescence complementation (BiFC) experiments, the coding sequence of the ASK18 was amplified with ASK18F and ASK18R primers and cloned into pBiFC2 (Kodama and Hu, 2013) vector (pBiF2_ASK18) using Gateway technology. Similarly, PHR1F and PHR1R primers PHR1 was amplified and cloned into pBiFC3 (Kodama and Hu, 2013) vector (pBiF3_PHR1).

4. Confocal Imaging

For intracellular fluorescence, we used a confocal multispectral system TCS SP5 with LAS AF v.2.3.6 software (Leica Microsystems) and a 363.0 1.20 water-immersion objective. To avoid emission spectra overlap, the sequential mode was used. GFP, propidium iodide, and FM4-64 were excited with a 488-nm argon laser line. Fluorescence was detected using a photomultiplier (GFP 505 to 546 nm, propidium iodide, and FM4-64 625 to 670 nm).

5. Determination of Luciferase Activity:

Luciferase activity (LA) levels were detected using the LB 960 microplate luminometer center system (Berthold Technologies) with MikronWin 2000 software. Plants were grown in +Pi medium for 7 d and transferred to -Pi medium for 2 d, and single plants were transferred to a microplate (Costar) containing Johnson medium. D-luciferin substrate (Sigma-Aldrich) was added to each well and the plate incubated (1 h) before As(V) supplementation. The plate was covered with optical film. The percentage of luciferase activity (%LUC activity) was calculated as $(\%LUC\ activity = [(LA_n - LA_0)/LA_0] * 100)$. Luciferase activity was determined in *N. benthamiana* using 1-cm discs of agroinfiltrated leaves transferred to a microplate as above. (Mohan TC et al., 2014. In transgenic *Arabidopsis* expressing *PHT1;1-LUC*, the luciferase activity was imaged using CCD camera with ChemiDoc XRS+ system, Bio Rad.

6. Northern blot and qRT-PCR Analysis

RNA was extracted with TRI Reagent® (Sigma). For Northern blot, 15 µg total RNA was loaded in each lane. RNA electrophoresis, transfer to nylon membranes and hybridization was done using standard procedures as described in Franco-Zorrilla et al (2005). The radioactive *PHT1;1* specific probe labeled with 40 uCi of α-32P-dCTP was obtained from Amersham Company.

For quantitative reverse transcription-PCR, the RNA extracted with TRI Reagent® (Sigma) was used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). The cDNA was diluted 10 times with water and qRT-PCR was performed using SYBR green reagent. Three independent biological samples were used for each treatment. Each sample was normalized using EF1a (for primer pairs, see table B).

7. Western Blot Analysis

Arabidopsis roots or agroinfiltrated *N. benthamiana* leaves were frozen and then homogenized in extraction buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1 % Np-40, 1 mM PMSF (Phenylmethylsulfonyl fluoride) and 1x complete protease inhibitor. The extracts were centrifuged twice at 4 °C for 15 minutes and supernatant was collected. Protein concentration in the supernatant was determined by Bradford assay (Bio-Rad). Protein samples were heated at 95°C for 5 minutes in 2XSDS loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol, 0.02 % bromophenol blue, 1 % β-mercaptoethanol) and 30 µg of protein was loaded per lane on 7.5 % SDS-PAGE gels (Sambrook et al., 1989). Using semidry blotting system proteins from gel were transferred to a nitrocellulose membrane and were then saturated with 5 % non-fat milk. Immuno detection of proteins was done using HRP conjugated antibodies, HA-HRP and GFP-HRP (Milttenyibiotec).

8. *In Vivo* Protein Degradation Assays

Seedlings were grown in Jonson solid medium for 7 d and then transferred to Jonson media lacking Pi for 3d and 30µM As(V) treatment was done as indicated in the results. The effect of proteasome inhibition was tested by adding 50 µM MG132 (Sigma-Aldrich) to the liquid Jonson medium lacking Pi. Root samples were harvested at specific time points as indicated. Protein extraction and immunoblotting were performed as mentioned above.

9. Chromatin immuno Precipitation (ChIP) Assay

ChIP assays in the WRKY6-GFP–overexpressing line and *N. benthamiana* were as described (Lee et al., 2007). OXWRKY6-GFP seedlings were grown on +Pi medium for 7 d and transferred to -Pi medium for 2 d; plants were then treated with 30 µM As(V) for 1.5 h. Leaves of *N. benthamiana* were agroinfiltrated with *PHT1;1p-LUC* wild type, a mutated version of *PHT1;1p-lm/lm-LUC* and WRKY6-GFP–overexpressing construct. As a negative control in both cases, we used leaves of *N. benthamiana* agroinfiltrated as above, replacing the WRKY6-GFP–overexpressing construct with a GFP-overexpressing construct.

Extracts of WRKY6-GFP–overexpressing seedlings (~2 g) or *N. benthamiana* leaves (~4 g) were immunoprecipitated with 40 µL anti-GFP Affinity Matrix (Abcam). Precipitated DNA was dissolved in 40 µL water, and 1 µL was used for qPCR. ACT2 was used as internal

control for the OXWRKY6-GFP–overexpressing line experiment. Arabidopsis Col-0 plants were grown on 1 mM Pi medium for 7 d, transferred to -Pi medium for 2 d and then to liquid -Pi medium alone or supplemented with 30 μ MAs(V). Tissue was fixed, and immunoprecipitated DNA was recovered using Chelex 100 resin (Bio-Rad; 10 g/100 mL double distilled water). All ChIP experiments were quantified by qPCR. Primers used to amplify the target gene fragments are shown in Table B.

10. Transcriptome Analyses of As(V) Response

Transcriptomic analyses were performed using the Affymetrix ATH1. For the Affymetrix ATH1 array, wild-type plants were grown on Johnson medium with 1 mM Pi for 7 d, transferred to -Pi medium for 2 d and then to medium supplemented with 30 μ M As(V) or 30 μ M Pi (8 h). RNA was isolated from three independent biological samples for each condition using the RNeasy plant mini kit (Qiagen). RNA amplification, microarray hybridization, and scanning were done as reported by Bustos et al., (2010).

11. Identification of *cis*-Regulatory Elements by *in Silico* Analysis.

To identify putative *cis*-acting regulatory elements, we identified promoter sequences (0.5 kb) of genes preferentially downregulated by As(V) in The Arabidopsis Information Resource (<http://www.Arabidopsis.org/>). This group of promoter sequences was submitted for overrepresented motifs analysis to Element (<http://element.mocklerlab.org/>). The motifs identified with $P < 0.05$ were used in further analysis.

12. Accession Numbers.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: WRKY6, AT1G62300; PHT1;1, AT5G43350; ASK18, AT1G10230; PHR1, AT4G28610. Array data from this article can be found in the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) database under accession number GSE49037.

13. Oligos used in this thesis (Table B)

ID	Name	Sequence	Name	Sequence
Binary Construct				
AT1G62300	WRKY6-GW-F	ggggacaagttgtacaaaaagcaggctcaatggacagaggatggtctg	WRKY6-GW-R	ggggaccactttgtacaagaagctgggtcctattgattttgtgttc
AT5G43350	PHT1;1p-I/II-F	cctgaaagcctcataaagaacaattgaagcaacatc	PHT1;1p-I/II-R	cctgaggatcctcttagagctctataatcataaac
AT5G43350	PHT1;1p-Im/II-F	ctggaaggcgtagatgaactcttagatgatgttttatac	PHT1;1p-Im/II-R	aaacatcatctaagagttcatctacgcctccagaatatg
AT5G43350	PHT1;1p-I/II-F	gggtgtcatattagttccttcgtatcgctccagcttag	PHT1;1p-I/II-R	agctggacgatacgaaggaaactaatgaacacctatac
AT5G43350	PHT1;1p-Im/II-F	gtataaggtgttcatattagttccttcgtatgaactcttagatgatgttttatac	PHT1;1p-Im/II-R	gtataaaacatcatctaagagttcatctacgaaggaaactaatgaacacctatac
AT1G10230	ASK18F	atggctcttcttccgaagag	ASK18R	ttactcattaaaagccaagca
AT4G28610	PHR1F	atggaggctcgctccagttcatag	PHR1R	tcaattatcgattttgggacgc
qRT-PCR				
AT1G18070	EF1a-F	cccaggctgattgtgctgt	EF1a-R	gggtgggtggcatcctctgtt
AT5G43350	PHT1;1-F	cctcaactctccagagaagttctta	PHT1;1-R	ttcggcatttcttagagc
AT1G62300	WRKY6-F	gcaacagcaacaacagaacaa	WRKY6-R	acacatctcggcctcac
AT4G33030	SOD1-F	gggactctcaacgttctctttg	SOD1-R	cccatcgctccaagtgtttac
AT1G78000	SULTR1;2F	atccgttttcaaacgagctc	SULTR1;2R	tcaagaatgatgcaccaatga
AT4G23100	GSH1F	ttccctgtctccctggtg	GSH1R	tccaagtatctctcaaccgaac
AT5G27380	GSH2F	cccagcatagcttaccatt	GSH2R	gcaatgtcctctttgtgtcc
AT2G02990	RNS1 F	ttgttatccaaattcaggcaaa	RNS1R	agttaggccaagaccatgaat
AT5G03545	At4F	tggccccaacaacaagag	At4R	cgaacattcacaatcataatctcc
AT5G44070	PCS1F	tcgatcctggagctaaatgg	PCS1R	ttcgagcaatccaacatt
AT2G21045	AtARQ1F	tttgatcgtggcttgaacg	AtARQ1R	ttagcccatggtcgtaccc
ChIP				
AT5G43350	AT5G43350 (ARE)-F	tcacccatctcacgtatcct	AT5G43350 (ARE)-R	tcacataactcctccgtatg
AT1G49240	ACT8-F	cccgcctataataatgttcaacac	ACT8-R	gacgacgaggcaattcaaaag
AT5G43350	AT5G43350-BF	aaggtgttcatattctggaag	AT5G43350-BR	gatgtttaagattttgaacacca
AT5G43350	AT5G43350m-BF	aaggtgttcatattagttcct	AT5G43350-BR	gatgtttaagattttgaacacca

RESULTS

1. Transcriptional Analysis of Pi Transporter *PHT1;1* in response As(V)

As(V) enter inside plant cells using the Pi transporter *PHT1;1*. To analyze the expression pattern of *PHT1;1* in response to As(V), we performed a northern-blot analysis using *Arabidopsis* seedlings exposed to 30 μ M As(V) or 30 μ M Pi for 8h. Results showed that the high affinity Pi transporter *PHT1;1* is more efficiently repressed by As(V) than does Pi (Figure 1A) though the Pi uptake rate is two times greater than that of As(V) (Catarcha et al., 2007). To understand the molecular mechanisms involve in the repression of *PHT1;1* by As(V), we generated a transcriptional reporter line that express luciferase gene driven by the promoter of *PHT1;1* (*PHT1;1-LUC*; Figure 1B). The functionality of this construct was verified by analyzing the *PHT1;1-LUC* activity in plants exposed to Pi and As(V). *PHT1;1-LUC* activity decreased more rapidly in response to As(V) than to Pi (Figure 1B). This observation re-confirmed our previous northern-blot results indicating that this construct serves as a suitable readout for As(V) repression of *PHT1;1*.

To verify if the repression observed in *PHT1;1-LUC* was derived from a possible As(V) toxicity we analyzed the luciferase activity in two constructs where the luciferase was fused to the promoters of two unrelated genes CIRCADIAN CLOCK ASSOCIATED1(*CCA1*) and CHLOROPHYLL A/B-BINDING PROTEIN (*CAB*) (*CCA1-LUC* and *CAB-LUC*, respectively). We found that As(V) had no effect on *CCA1-LUC* and *CAB-LUC* promoter fusions (Figure 1C). Dose-response experiments using increasing As(V) concentrations showed that *PHT1;1-LUC* repression occurred in a dose dependent manner and it is more rapid at high As(V) concentration. *PHT1;1-LUC* activity was reduced by >40% after 2 h of exposition to 30 and 100 μ M of AsV (Figure 1D). After this rapid repression, a recovery in the *PHT1;1-LUC* activity was observed even in the presence of As(V). This recovery does not occur at high As(V) concentration. The repression/recovery pattern obtained with the *PHT1;1-LUC* transgenic plants was validated by RT-PCR (Figure 1E). Therefore, plants exhibits a sensitive mechanisms for As(V) perception that we decided to explore further.

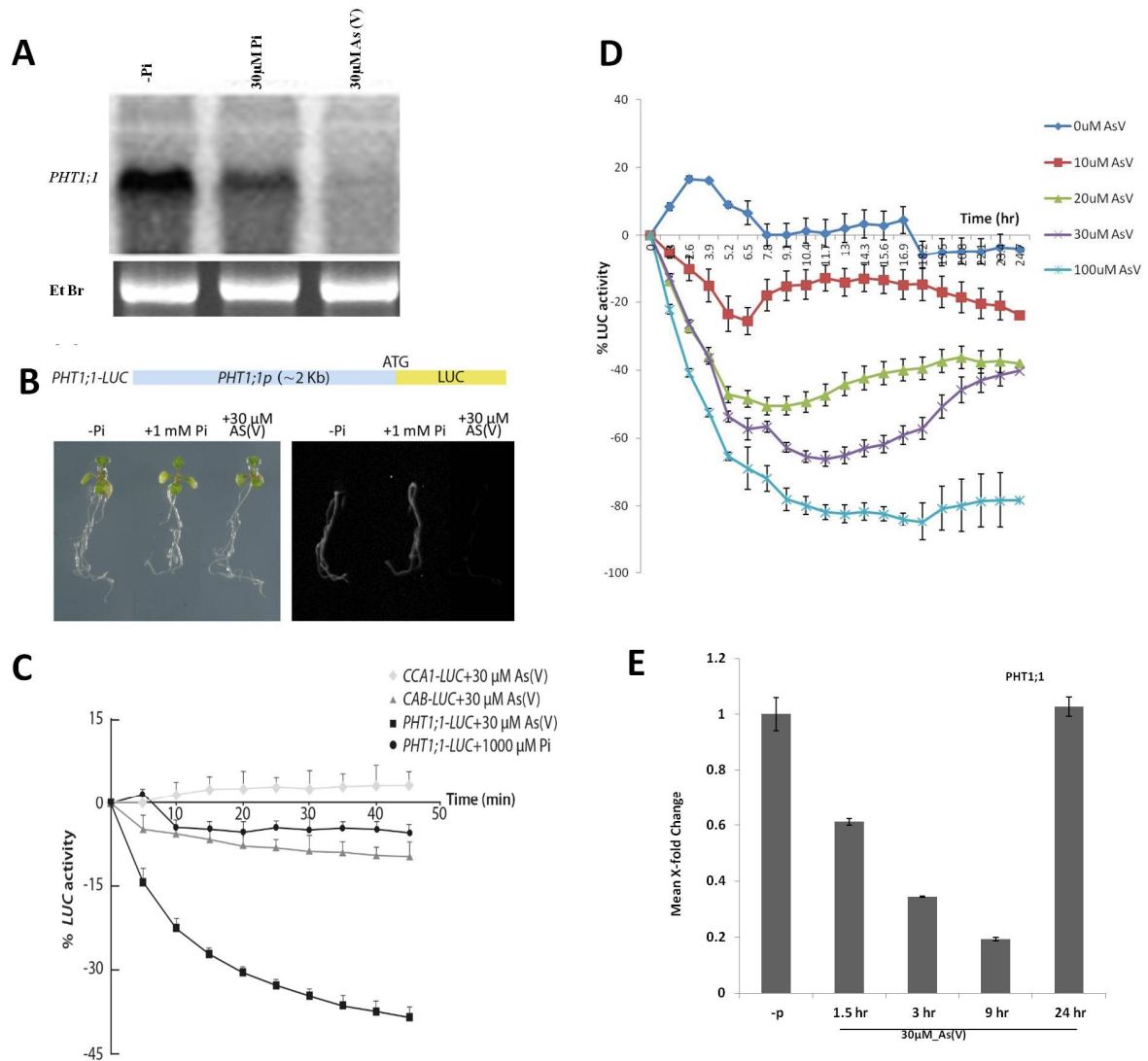


Figure 1: Arsenate Represses the Pi Transporter *PHT1;1*.

- Northern-blot analysis of *PHT1;1* expression in response to As(V) and Pi. Col-0 plants were grown on 1mM Pi medium for 7 day , transferred to -Pi medium for 3 d, and then treated with 30μM AsV or 30μM Pi for 8 hrs.
- Diagram of the 2-kb *PHT1;1* promoter region fused to the luciferase reporter gene (*PHT1;1*-LUC; top). Analysis of LUC activity (bottom) in transgenic plants expressing *PHT1;1*-LUC grown on 1 mM phosphate medium for 7 d, transferred to -Pi medium for 2 d, and finally to -Pi medium supplemented with 30 μM As(V) or 1 mM Pi for 16 h.
- Kinetic study of LUC activity in response to 30 μM As(V) or 1 mM Pi in *PHT1;1*-LUC–expressing plants and in control transgenic lines *CCA1*-LUC and *CAB*-LUC. Values show mean ± SD.
- Kinetic study of LUC activity in response to different concentration of As(V) in *PHT1;1*-LUC–expressing plants. Values show mean ±SE (n=16).
- qRT-PCR expression analysis of *PHT1;1*. Col-0 plants were grown on 1 mM phosphate medium for 7 day, transferred to -Pi medium for 3 d, and then treated with 30μM As(V) in Pi lacking liquid media for different time course. Values show mean ±SD (n=3)

2. Arsenate Delocalize the Pi transporter PHT1;1

As we mentioned in the introduction Pi-transporters are delocalized in response to Pi (Lundh et al., 2009; Bayle et al 2011). In order to explore if As(V) affects the localization of the Pi transporter we exposed *PHT1;1*-GFP (Green fluorescent protein) over-expressing plants (Gonzalez et al 2005) to 30 μ M As(V). During 1.5 hours we observed that *PHT1;1* repression in response to As(V) is also accompanied by endocytosis of the As(V)/Pi transporter from the plasma membrane (Figure 2A).

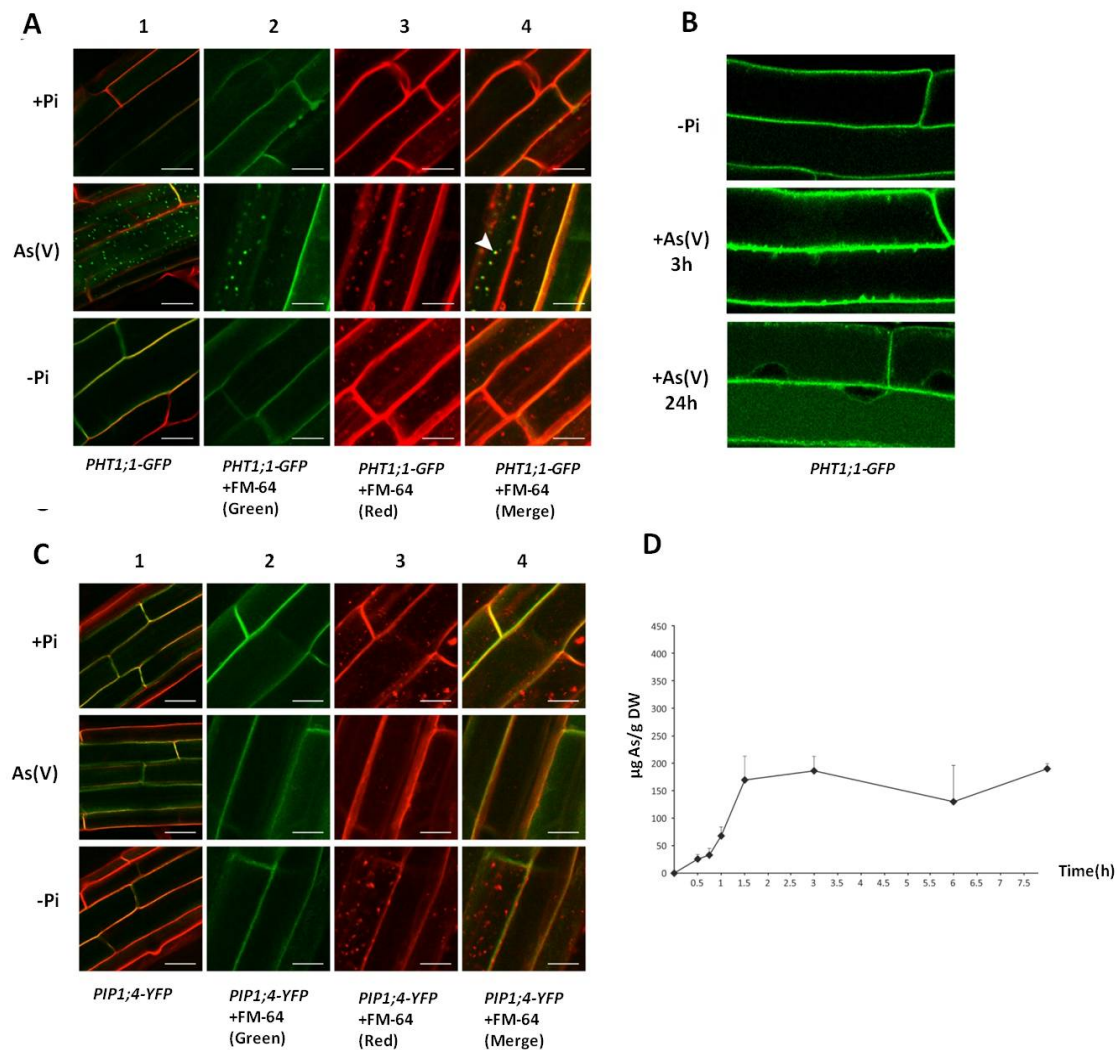


Figure 2: Delocalization of the Pi transporter is an As(V)-specific response that correlates with arrest of intracellular arsenic accumulation. (A) (B) & (C) Confocal analysis of *PHT1;1*-GFP-expressing Arabidopsis root epidermal cells.

A) Five-day-old plants grown in -Pi medium were transferred to fresh -Pi medium or to medium containing 1 mM Pi (+Pi) or 30 μ M As(V) [+As(V)] for 1.5h in the dark. Roots were stained with

- propidium iodide (column 1) or with the endocytic tracer FM4-64 (columns 2 to 4). Arrowhead indicates colocalization of FM4-64 with PHT1;1-GFP in endosomes. Bars = 10 μ m.
- B) Five-day-old *PHT1;1-GFP*-expressing plants grown in -Pi medium were incubated (24 h) (-Pi) or exposed to 30 μ M As(V) for 3 h [+As(V) 3 h] and 24 h [+As(V) 24 h] in the dark.
 - C) Confocal analysis of *PIP1;4-YFP*-expressing Arabidopsis root epidermal cells. Five-day-old plants grown in -Pi medium were exposed to 1 mM Pi (+Pi) or 30 μ M As(V) (+As(V)) for 1.5 h in the dark. Roots were stained with propidium iodide (column 1) or with the endocytic tracer FM4-64 (columns 2-4). Bars = 10 μ m.
 - D) Time course analysis of the intracellular arsenic concentration in wild-type plants exposed to 5 μ M As(V). Values show mean \pm SD (n=3).

After 24 hours of incubation with As(V), GFP fluorescence labeled the vacuole lumen (Figure 2B), indicating PHT1;1-GFP internalization from the plasma membrane and transport through the endocytic pathway for turnover in the vacuole.

To analyze if the Pi transporter de-localization is caused by As(V) toxicity we exposed plants over-expressing the aquaporin PIP1;4 fused to the yellow fluorescent protein (PIP1;4-YFP) to As(V) for 1.5h. This protein localized in the plasma membrane as PHT1;1. We found that As(V) did not alter the PIP1;4-YFP protein localization at the plasma membrane (Figure 2C), indicating that the delocalization of PHT1;1 is not a nonspecific response to As(V) toxicity. Since As(V) provokes a rapid delocalization and transcriptional repression of PHT1;1 we studied the kinetic accumulation of arsenic in Col-0 plants at short times of exposure. We observed an intracellular arrest of As(V) content after 1.5h (Figure 2D). This finding correlated with delocalization/repression of PHT1;1 suggesting that plants restricts the As(V) uptake via two parallel responses: transcriptional downregulation and delocalization of PHT1;1.

To prove that the rapid delocalization of PHT1;1-GFP from the plasma membrane is As(V) dependent, we exposed PHT1;1-GFP over-expressing plants to As(V) for 2h and then As(V) was removed from the media. We found that As(V) removal lead to restoration of PHT1;1 localization at the plasma membrane (Figure 3A). After a second round of As(V) exposure, plants again showed the delocalization of PHT1;1-GFP in endosomes (Figure 3A). Using the same strategy, we treated PHT1;1-LUC plants with different rounds of +/- As(V) exposure and the analysis of the luciferase activity showed a transcriptional repression/reactivation pattern (Figure 3B) closely linked to PHT1;1-GFP delocalization/relocalization at the plasma membrane (Figure 3A). Using qRT-PCR

experiments we confirmed that the *PHT1;1*-LUC pattern expression mimicry of *PHT1;1* transcript accumulation (Figure 3C). The rapid response of the system under Pi-free conditions indicated that both *PHT1;1* transcriptional repression and membrane delocalization depend exclusively on As(V).

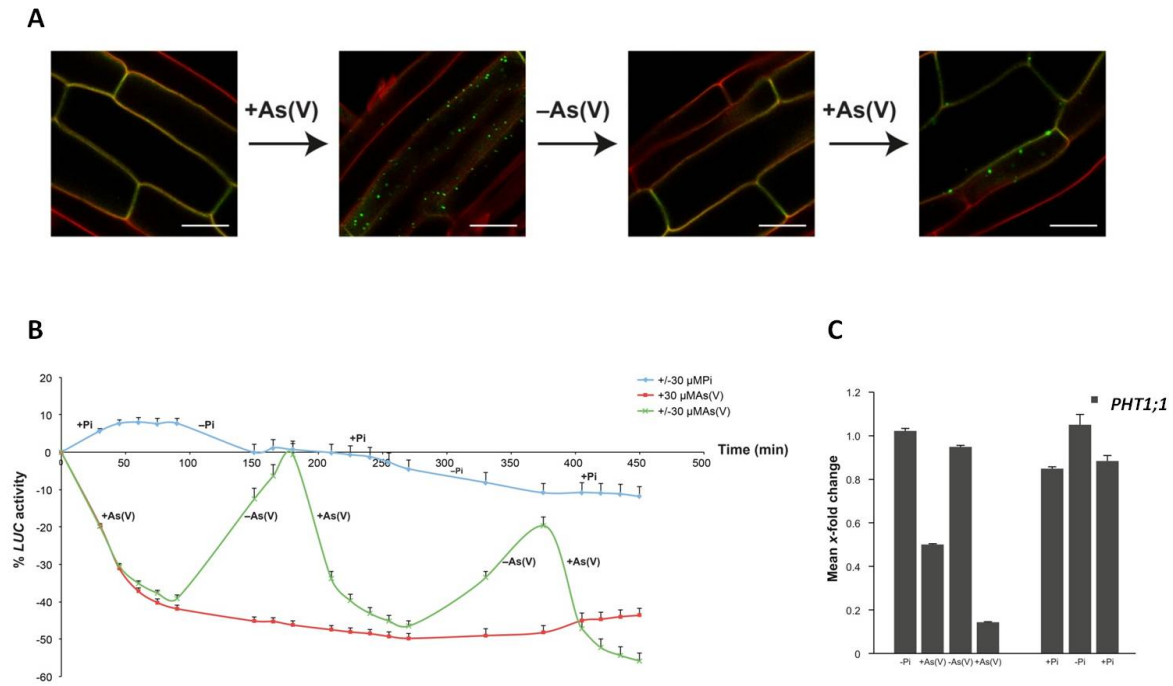


Figure 3: Repression and Delocalization of *PHT1;1* is As(V) Specific.

- A) Analysis of *PHT1;1*-GFP localization after two pulses of 30 μ M As(V) [\pm As(V)] in *PHT1;1*-GFP-expressing Arabidopsis root cells. Duration of each pulse and gaps between them were 1.5 h in the conditions as in (A). Bars = 10 μ m.
- B) Kinetic study of LUC activity in response to pulses of 30 μ M Pi (\pm Pi; blue line), 30 μ M As(V) [\pm As(V); green line], or to a continuous concentration of 30 μ M As(V) (red line) in *PHT1;1*-LUC-expressing plants. Duration of each pulse and gaps between them were 1.5 h; during the gap, samples were washed with buffer to remove Pi and As(V) from the medium. Values show mean \pm SD.
- C) qRT-PCR kinetics expression analysis of *PHT1;1* gene in response to As(V) and Pi pulses. Kinetic study of *PHT1;1* expression in response to pulses of 30 μ M As(V) (\pm As(V)) or 30 μ M Pi (\pm Pi). Duration of each pulse and gaps between them were 1.5 h; during the gap. Values show mean \pm SD.

3. WRKY6 TF mediates *PHT1;1* repression, in Response to As(V)

To further study the role of As(V) in the repression of Pi starvation- induced genes similar to *PHT1;1*, we performed a transcriptomic analysis using Affymetrix ATH1 array. For this purpose RNA samples were collected from Pi-starved plants exposed to As(V) or Pi for 8 h. The analysis of transcriptomic data showed that only 10% of genes up regulated by Pi starvation were repressed more efficiently by As(V) than by Pi. This finding indicates

that few genes show the transcriptional repression pattern of the Pi transporters in response to As(V) (Figure 4A). Using the promoter regions of these genes, we performed an *in silico* analysis of enriched sequences to identify relevant regulatory elements for As(V)-signaling. This approach allowed identification of a putative *cis* As(V) repression element (ARE; KTCCAG, K:G/T) also found in the promoter region of AsV/Pi transporter *PHT1;1* (Figure 4B). For the functional characterization of this ARE element we designed different versions of *PHT1;1* promoter fused to luciferase gene. We used four versions: one containing the two -wild-type ARE elements present in the promoter, and others three derived from the sequential mutation of each element as shown in figure 4C. We developed transgenic lines over-expressing these ARE-mutated versions of *PHT1;1-LUC*. Analysis of luciferase activity in these transgenic plants showed that, in the promoter version containing both ARE mutation, the luciferase activity was less compare to the rest of versions. This results shows that ARE element (KTCCAG), contributes to repression of the Pi transporter in response to As(V) (Figure 4D).

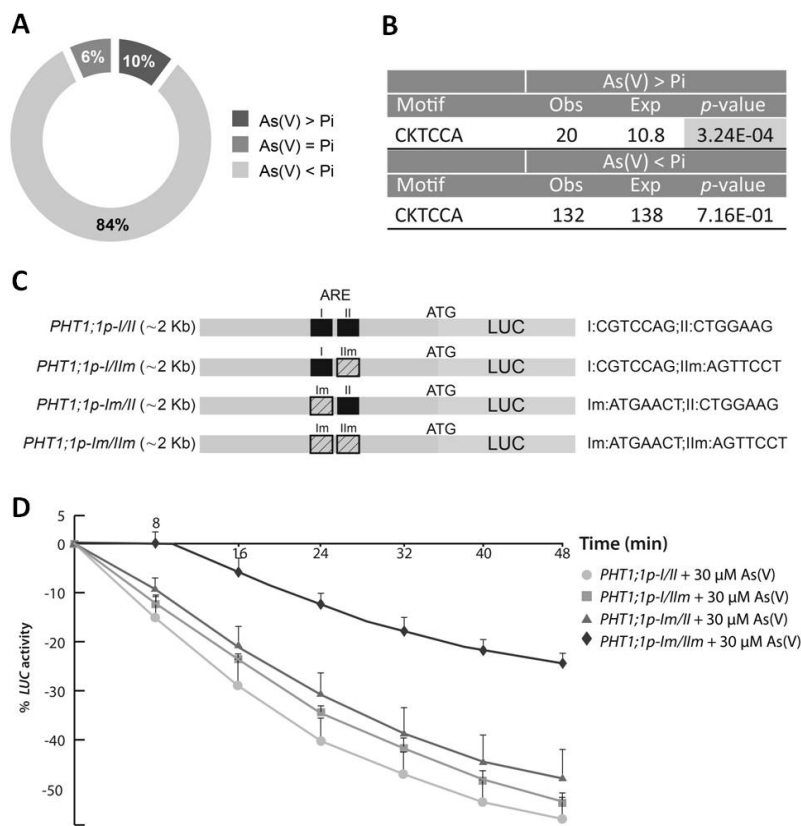


Figure 4: The ARE Contributes to Down-regulation of the Pi Transporter in Response to As(V).

(A) Diagram showing the relative sizes of the three classes of down regulated genes in response to 30 μM As(V) and 30 μM Pi identified in a microarray analysis. As(V) > Pi, genes preferentially down-regulated by As(V); As(V) < Pi, genes preferentially down-regulated by Pi; As(V) = Pi, genes down-regulated equally in response to both stimulus. For microarray analysis, wild-type plants were grown on Johnson medium with 1 mM Pi for 7 d, transferred to -Pi for 2 d, and finally to -Pi medium supplemented with 30 μM As(V) or 30 μM Pi (8 h).

(B) Analysis of ARE frequency in 0.5 kb of the promoter regions of genes in the As(V)>Pi and As(V)<Pi classes. Table shows observed and expected ARE numbers assuming random distribution. Significant overrepresentation is highlighted (x² test, P < 0.05).

(C) Diagram showing wild-type (PHT1;1p-I/II) and mutated versions (PHT1;1p-I/II_m, PHT1;1p-I_m/II, and PHT1;1p-I_m/II_m) of the *PHT1;1* promoter region fused to luciferase (*PHT1;1*-LUC). The ARE in the *PHT1;1* promoter (I and II; black) was mutated sequentially (I_m and II_m; diagonal stripes) by PCR site directed mutagenesis.

(D) Kinetic analysis of LUC activity in transgenic plants expressing the PHT1;1p-I/II, PHT1;1p-I/II_m, PHT1;1p-I_m/II, or PHT1;1p-I_m/II_m constructs, in response to 30 μ M As(V) (1.5 h). Values represent data from analysis of 12 independent lines of each construct; mean \pm SD.

In order to identify the transcription factor binds ARE element we scanned its sequence looking for any regulatory box known. We found that ARE box is similar to TTTTCCAC (WK-box) core motif that, in addition to the classical W-box, is bound by plant WRKY transcription factors (Rushton et al., 2010). The analysis of our microarray profile using plants exposed to As(V) for 8h identified a WRKY family gene, WRKY6, which is induced by As(V). It is reported that WRKY6 regulates several plant responses, like nutrient starvation (Robatzek and Somssich, 2002; Kasajima et al., 2010), and also negatively regulates PHO1, a plasma membrane protein, which is involved in xylem Pi loading (Chen et al., 2009). These observations prompted us to consider WRKY6 involvement in *PHT1;1* repression during As(V) response.

To test whether WRKY6 is responsible for *PHT1;1* repression following As(V) stress, we evaluated the kinetics of *WRKY6* in response to As(V). qRT-PCR analysis showed that the transcript accumulation of *WRKY6* in response to As(V) was inverse to that of As(V)/Pi transporter *PHT1;1* repression by As(V) (Figure 5A). In response to As(V) pulses, the *WRKY6* activation/repression pattern was again inverse to that of *PHT1;1* as predicted for a transport repressor-mediated mechanism (Figure 5B). However, the expression of *WRKY6* in response to high/low Pi concentrations was unaltered in response to Pi pulses (Figure 5B).

To further characterize the role of *WRKY6* in *PHT1;1* repression, we have generated the transgenic *Arabidopsis* line over-expressing *WRKY6* fused to GFP (*WRKY6*-GFP) and also we identified the knock-out line of *wrky6*. Using qRT-PCR we analyzed the level of expression of *PHT1;1* in wild-type, *wrky6* and *WRKY6* over-expressing plants exposed or not, to As(V). We found that in the *Arabidopsis* line expressing *WRKY6*-GFP, in the absence of As(V), the *PHT1;1* expression was similar to that of wild-type plants exposed to As(V). In the case of *wrky6* line, the expression of *PHT1;1* was unaltered (Figure 5C). These results

support the idea that *WRKY6* mediates *PHT1;1* transcriptional repression during As(V) stress. To analyze if *WRKY6* represses specifically the expression of *PHT1;1* we analyze the transcript accumulation of *SQD1*, a gene induced by Pi starvation and not preferentially repressed by As(V). We found that the expression of this gene was unaltered in the *WRKY6*-GFP-overexpressing plants (Figure 5D) which strongly supports the specific role of *WRKY6* in the repression the Pi-transporter *PHT1;1*.

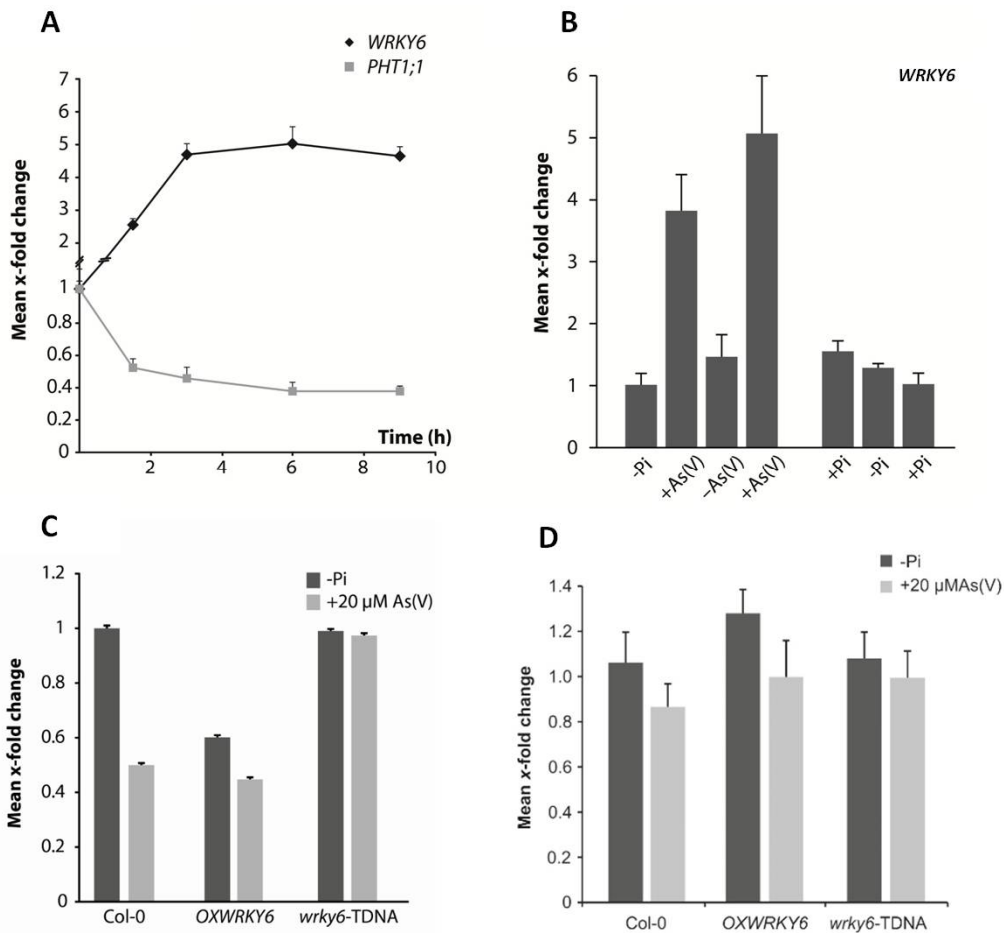


Figure 5: *WRKY6* Responds to As(V) and Represses the Pi Transporter *PHT1;1*.

- Kinetic study of *WRKY6* and *PHT1;1* expression by qRT-PCR in wild-type plants exposed to 30μM As(V). Values show mean ±SD.
- qRT-PCR expression analysis of *WRKY6* in wild-type plants in response to 30 μM As(V) pulses [\pm As(V)] or in response to 30μM Pi pulses (\pm Pi); duration of each pulse and gap was 1.5 h. Values show mean ± SD.
- qRT-PCR of *PHT1;1* transcript in wild-type plants (Col-0), in the *WRKY6*-GFP-overexpressing line (*OXWRKY6*), and in *wrky6*-TDNA line grown in +Pi medium for 7 d, transferred to -Pi for 3 d and then to -Pi medium alone or with 20 μM As(V) (1.5 h). In the case of *WRKY6*-overexpressing lines, values show data from analysis of 10 independent lines. Values show mean ±SD.
- qRT-PCR expression analysis of *SQD1*: as explained in (C).

4. ARE mediates WRKY6 Repression of the Pi transporter *PHT1;1*

To test whether WRKY6 interacts directly with the *PHT1;1* promoter region *in vivo*, we performed Chromatin ImmunoPrecipitation (ChIP) assays using *WRKY6*-GFP overexpressing line. Quantitative PCR (qPCR) of chromatin fragments from this line immunoprecipitated with anti-GFP showed enrichment in ARE-bearing fragments of the *PHT1;1* promoter compared with wild-type plants or the *ACTIN8* (*ACT8*) control (Figure 6A). To further demonstrate that ARE mediates WRKY6 repression of the Pi transporter *PHT1;1*, we agroinfiltrated *PHT1;1*-LUC or *PHT1;1*-LUC ARE mutants versions in *N. benthamiana* with or without *WRKY6*-GFP. The analysis of transient LUC activity expression assays using leaf discs shows that WRKY6 was able to repress all constructs containing at least one functional ARE (Figure 6B). But in the case of the *PHT1;1*-LUC with both ARE mutated WRKY6 repressor capacity was fully impaired (Figure 6B).

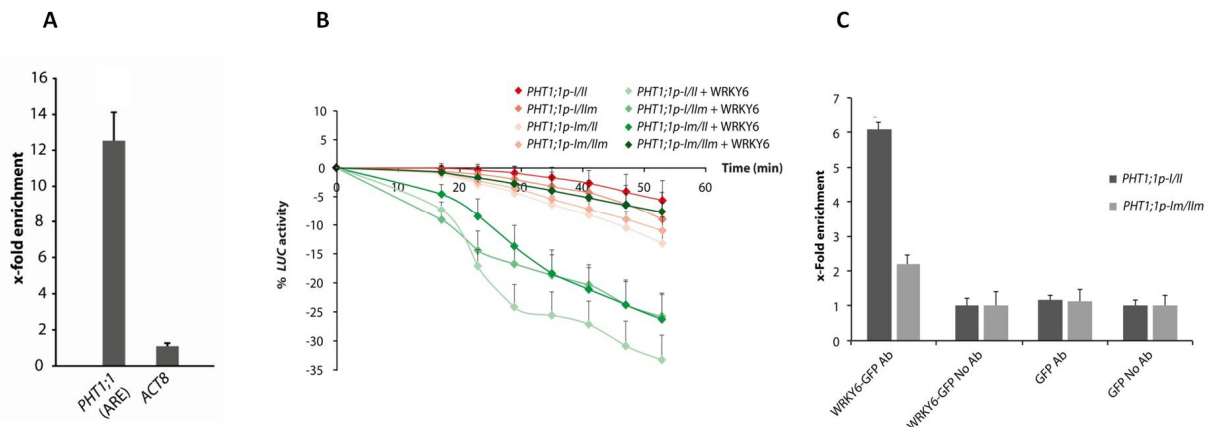


Figure 6: The ARE Mediates WRKY6 dependent Repression of the Pi Transporter *PHT1;1*.

- ChIP assay of *WRKY6*-GFP seedlings and *PHT1;1* promoter PCR amplification analysis. qPCR of ARE-containing fragments of the *PHT1;1* promoter. Enrichment was calculated relative to wild-type plants. *ACT8* was used as negative control. Values show mean \pm SD.
- Kinetic analysis of transient LUC activity in *N. benthamiana* leaf discs agroinfiltrated with *PHT1;1*-LUC wild-type or the mutated versions alone or with a *WRKY6*-GFP-overexpressing construct. Leaf discs were incubated in medium with 30 μ M As(V) (1.5 h). Values show mean \pm SD.
- ChIP assay of *WRKY6*-GFP followed by qPCR of the *PHT1;1* promoter. ChIP assays were performed in *N. benthamiana* leaf discs agroinfiltrated with *PHT1;1*-LUC wild type and the *PHT1;1*-LUC mutated version (*PHT1;1*-LUCm), with *WRKY6*-GFP or GFP-overexpressing constructs. Values represent the x-fold enrichment of *WRKY6*-bound DNA of the *PHT1;1* promoter in immunoprecipitated samples relative to total input DNA. ARE (*PHT1;1*-LUC) or mutated ARE-containing fragments (*PHT1;1*-LUCm) in the *PHT1;1* promoter were amplified by qPCR using specific primers. Values show mean \pm SD.

These results indicate that ARE *cis* motif mediates WRKY6 repression of the Pi transporter. To test if ARE sequence are important for the binding between WRKY6 and *PHT1;1* promoter we performed ChIP assays using *N. benthamiana* plants infiltrated with *PHT1;1*-LUC version, *PHT1;1* ARE mutant and WRKY6-GFP or GFP alone. After the immunoprecipitation using anti-GFP and DNA fragment purification, qPCR shows that there are less enrichment in the fragment of DNA containing the ARE mutated versions compared to the wild-type and there are no enrichment in the control samples used. This results showed that WRKY6 requires an intact ARE to bind the *PHT1;1* promoter region (Figure 6C).

5. WRKY6 over-expressing line shows As(V)-Tolerance Phenotype

In order to analyze if the WRKY6 repression of the Pi transporter *PHT1;1* confers any As(V)-tolerance phenotype, we grown wild-type plants, *WRKY6*-GFP over-expressing line and *wrky6* mutant with As(V) or not. We found that As(V) tolerance was markedly enhanced in the *WRKY6*-GFP-overexpressing line (Figure 7A); quantification of root length showed a significant increase compared with wild-type plants in the presence of As(V) (Figure 7B). No differences in root length were observed in the *wrky6*-TDNA insertion line relative to wild-type plants, probably due to functional redundancy among WRKY gene family members. In the absence of As(V), the *WRKY6*-GFP-overexpressing line and *wrky6*-TDNA insertion line showed no visible differences in root length compared with wild-type plants (Figure 7C). To further study the tolerant phenotype exhibited by the *WRKY6*-GFP-over expressing lines, we decided to quantify arsenic accumulation in wild-type plants, *WRKY6*-GFP-overexpressing line and *wrky6*-TDNA. Arsenic accumulation was increased in the *WRKY6*-overexpressing line compared with wild-type controls, whereas levels in the *wrky6*-TDNA mutant were significantly lower (Figure 7D). Reduction in As(V) uptake is associated with enhanced arsenic accumulation (Catarcha et al., 2007) supporting that WRKY6 modulates As(V) uptake through transcriptional repression of the As(V)/Pi transporters.

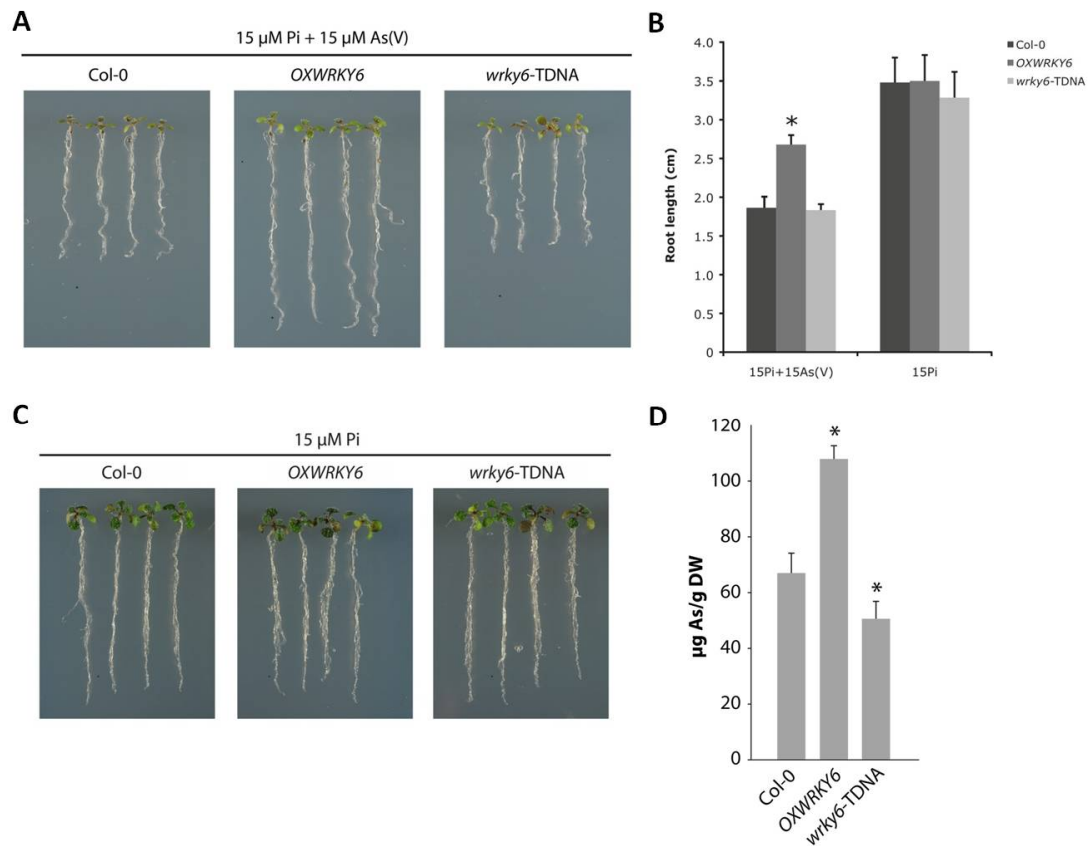


Figure 7: *WRKY6* over-expressing line shows As(V) tolerant phenotype and accumulates more arsenic.

- A) As(V) tolerance phenotype of wild-type (Col-0), *WRKY6*-GFP-overexpressing line (*OXWRKY6*), and *wrky6*-TDNA plants grown on 15 μ M Pi supplemented with 15 μ M As(V) for 7 d.
- B) Quantification of root length of wild-type (Col-0), *OXWRKY6* and *wrky6*-TDNA plants grown in 15 μ M Pi medium alone or with 15 μ M As(V) (7 days). Values show mean \pm SD. * p < 0.05; Student's *t*-test.
- C) Seedling phenotypes of wild-type (Col-0), *OXWRKY6* and *wrky6*-TDNA plants grown on 15 μ M Pi (7 days).
- D) Intracellular arsenic concentration in Col-0, *OXWRKY6*, and *wrky6*-TDNA plants exposed to 5 μ M As(V) (24 h). Values show mean \pm SD. * p < 0.05 (Student's *t* test). DW: dry weight.

6. Reactivation of *PHT1;1* expression in response to As(V) depends on As(V) concentration and detoxification machinery.

One surprising observation is that *PHT1;1* starts to recover its expression in the presence of As(V) during the 24h exposure (Figure 1D and Figure 8A), and this reactivation of *PHT1;1* expression depends on As(V) concentration (Figure 8A). One intriguing possibility would be that plants might recover As(V) uptake once they adapted to detoxify As(V). To explore this possibility, we decided to study if expression of *PHT1;1* recovery

depends on plant's detoxification capacity. Toward this, we analyzed the kinetic of expression of *PHT1;1* in wild-type plants exposed to As(V) in the presence of buthionine sulfoximine (BSO), an inhibitor of γ -glutamylcysteine synthetase, a key enzyme in the synthesis of glutathione, an essential component for As(V) detoxification. We found that the kinetic of the *PHT1;1* repression is unaltered in presence of BSO. In contrast, the recovery of *PHT1;1* expression is compromised in the presence of inhibitor (Figure 8B). To confirm this result we analyzed the expression of *PHT1;1* in response to As(V) in two mutants impaired in the synthesis of phytochelatin: *pad2-1* with low level of glutathione and *cad1-3*, deficient in phytochelatin synthase. The qRT-PCR analysis shows that the recovery of *PHT1;1* expression after As(V) exposition is altered in these mutants compared to wild-type plants (Figure 8C). These results strongly suggest that the reactivation of *PHT1;1* expression during As(V) exposition is tightly coordinated by concentration of As(V) and plant detoxification capacity.

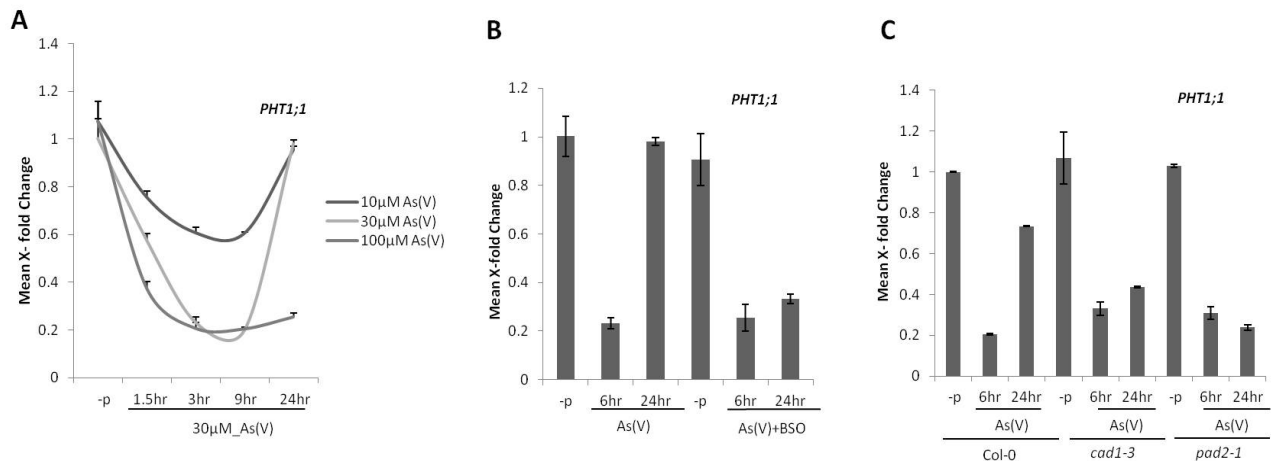


Figure 8: The recovery of the *PHT1;1* expression, in response to As(V) depends on As(V) concentration and detoxification capacity of plant. (A) to (C) qRT-PCR analysis of *PHT1;1* expression.

- Wild-type plants (Col-0) grown in +Pi medium for 7 d, transferred to -Pi for 3 d and then to -Pi medium alone or with 10, 30 and 100 μ M As(V) at different time. Values show mean \pm SD (n=3).
- Wild-type plants (Col-0) grown in +Pi medium for 7 d, transferred to -Pi or -Pi supplemented with 50 μ M BSO for 3 d and then to -Pi medium alone, with 30 μ M As(V) or 30 μ M As(V) and 100 μ M BSO for indicated time. Values show mean \pm SD (n=3).
- Wild-type plants (Col-0), *cad1-3*, and *pad2-1*, mutants were grown in +Pi medium for 7 d, transferred to -Pi 3 d and then to -Pi medium alone or with 30 μ M As (V) for indicated time. Values show mean \pm SD (n=3)

7. The reactivation of *PHT1;1* expression in response to As(V) requires active PHR1 transcription factor

During Pi starvation, *PHT1;1* transporter is induced under control of PHR1 transcription factor (Bustos et al., 2010; Rubio et al., 2001). We studied if PHR1 is relevant for the reactivation of *PHT1;1* expression after As(V) exposition. To this end, we analyzed *PHT1;1* expression in the *phr1* mutant background. As shown in Figure 9A the kinetics of *PHT1;1* repression by As(V) is the same as in wild-type plants. By the contrary, *PHT1;1* is unable to recover its expression in the *phr1* mutant at late hours of As(V) exposure (Figure 9A). This result indicate that PHR1 transcription factor is crucial for *PHT1;1* recovery during arsenate response. To gain more insight into the role of PHR1 in the As(V) response, we studied the stability of the PHR1 protein in presence of As(V). Western-blot analysis using protein extracts isolated from a transgenic line over-expressing GR:PHR1-HA (PHR1 fused to rat glucocorticoid receptor domain) exposed to As(V) at different time show that PHR1 is rapidly degraded in presence of As(V) followed by a recovery to its initial levels (Figure 9B). The rapid degradation of PHR1 and later recovery correlates with the kinetic of expression of *PHT1;1* in response to As(V) (Figure 9A). Furthermore, PHR1 was not degraded in response to Pi suggesting that PHR1 degradation is As(V) specific (Figure 9C).

We found that *PHT1;1* expression is not reactivated in response to As(V) in the GR:PHR1-HA overexpressing line (Figure 9D) suggesting that the recovered PHR1 may not be active or it may require other component or modifications or must be due to a secondary effect of overexpression. In order to obtain some clues about the mechanism behind the rapid degradation of PHR1 we analysed PHR1 stability, in the presence of MG132, an inhibitor of proteasome complex. We observed the stabilization of PHR1 in the presence of MG132, (Figure 9C). These results show that PHR1 degradation in response to As(V) occurs via proteasome. We have shown above that recovery of *PHT1;1* expression is altered by BSO and depends on a functional PHR1 (Figure 8B and 9A). We thus decided to examine the effect of BSO on PHR1 reestablishment. Our western blot analysis shows that pre treatment of plants with BSO abolished the PHR1 recovery during arsenic exposure (Figure 9E). This result indicates that PHR1 stability is regulated by detoxification capacity

of the plant system which in turn regulates the *PHT1;1* reestablishment after repression by As(V).

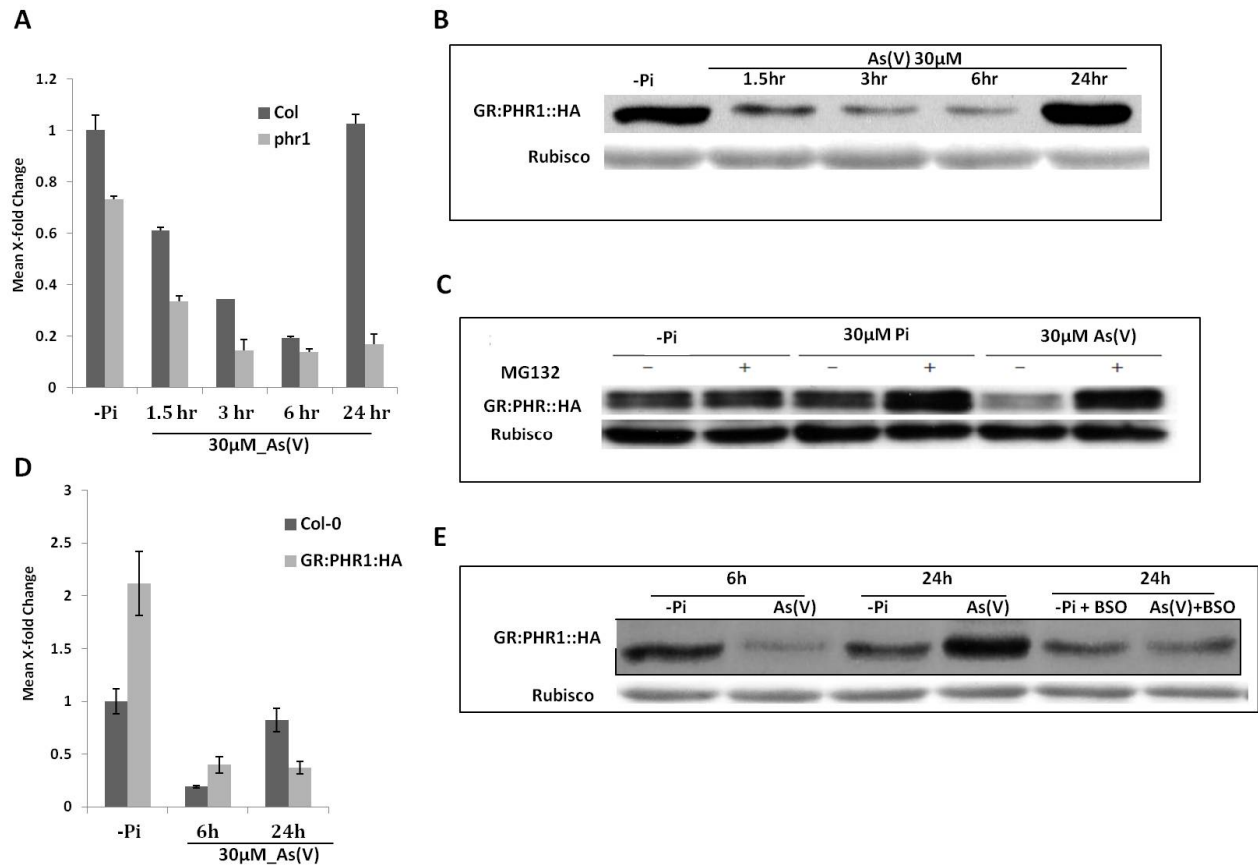


Figure 9: *PHR1* is essential for *PHT1;1* reactivation during As(V) exposure and As(V) induces the degradation of *PHR1* via proteasome pathway

- qRT-PCR analysis of *PHT1;1*: Wild-type plants (Col-0) and *phr1* were grown in +Pi medium for 7 d, transferred to -Pi 3 d and then to -Pi medium alone or with 30 μM As (V) for indicated timings. Values show mean ±SD (n=3).
- Western-blot analysis of *PHR1* degradation in response to As(V). Arabidopsis transgenic line over-expressing GR:PHR1-HA were grown on +Pi Johnson media for 7d, transferred to -Pi media with 5μM Dexamethasone for 3 d, then treated with 30 μM As(V) for indicated timings. Roots were collected for analysis. Rubisco was used as loading control.
- Western blot showing As(V) induced degradation of *PHR1* is stabilized by proteasome inhibitor MG132. Arabidopsis over-expressing GR:PHR1-HA were incubated with (+) or without (-) 50 μM MG132 along with -Pi, 30 μM Pi or 30μM As(V) for 6 hrs. Roots were collected for analysis. Rubisco was used as loading control.
- Western blot showing effect of BSO on reactivation of *PHR1* during arsenic stress. Arabidopsis transgenic line over-expressing GR:PHR1-HA were grown on +Pi Johnson media for 7d, transferred to -Pi media with 5μM Dexamethasone supplemented with or without 100μM BSO for 3 d, then treated with 30 μM As(V) with or without 100μM BSO for indicated timings. Rubisco was used as loading control.

- E) qRT-PCR analysis of *PHT1;1*: Wild-type plants (Col-0) and GR:PHR1:HA were grown in +Pi medium for 7d, transferred to -Pi 3 d and then to -Pi medium alone or with 30 μ M As (V) for indicated time. Values show mean \pm SD (n=3).

8. ASK18 is involved in PHR1 degradation in response to As(V)

To identify the molecular mechanism underlying PHR1 degradation in response to As(V) we analysed our previous results of transcriptomic analysis using plants exposed to 30 μ M As(V) for 8 hours (Castrillo et al., 2013). We found that only 32 genes were induced by As(V) but not by +Pi (1mM) or -Pi or 30 μ M Pi (Figure 10A). Out of these 32 genes we found one gene that encodes for a skp1 like protein named *ASK18* (AT1G10230) (Figure 10A). ASK18 is a part of SCF E3 ubiquitin ligase complex and therefore it is a good candidate to be involved in PHR1 degradation in response to As(V). We found that ASK18 does not interact with PHR1 based on yeast two hybrid assays, maybe because an F-box is required to bridge the interaction between ASK18 and PHR1 (data not shown). However, bimolecular complementation (BiFC) assays in *N. benthamiana* shows that ASK18 indeed physically interacts with PHR1 in the nucleus, probably using an endogenous F-box from *N. benthamiana* (Figure 10B).

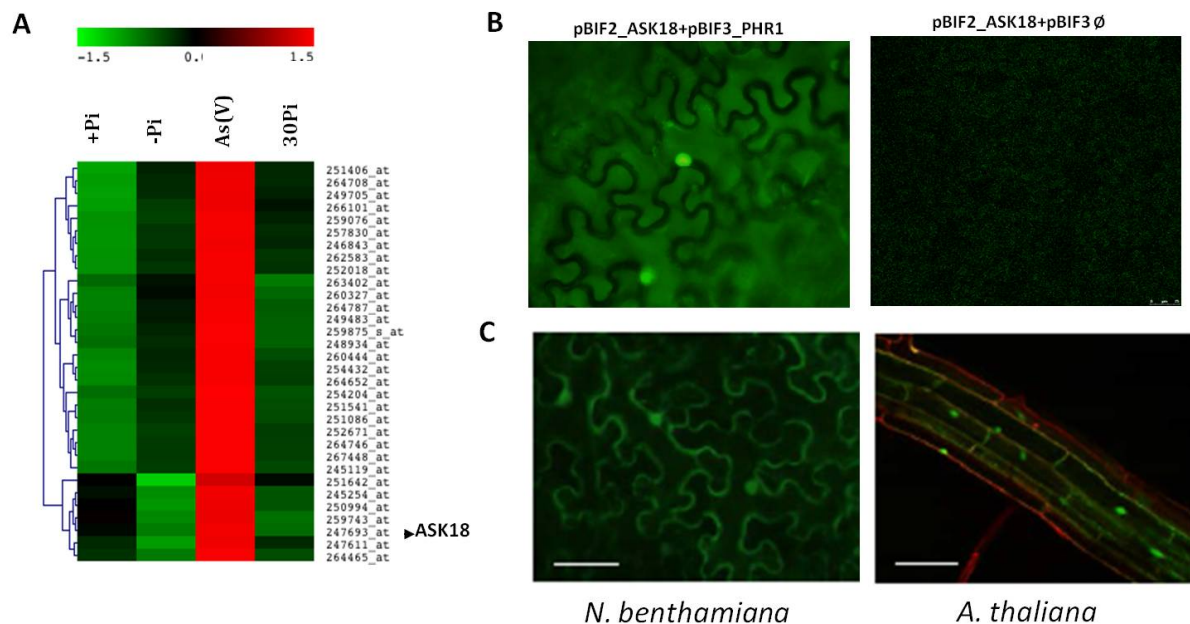


Figure 10: ASK18 is specifically induced by As(V) and interacts with PHR1 in the nucleus.

- A) Heat map generated from microarray data. Col-0 plants were grown on +Pi for 7d and transferred to -Pi for 3 days then exposed to 1mM Pi (+Pi), -Pi, 30 μ M As(V) or 30 μ M Pi for 8h. Genes specifically induced by As(V) were selected to generate heat map.

- B) Bimolecular Fluorescence Complementation assays show the interaction between ASK18 and PHR1. *Agrobacterium* carrying ASK18 fused to N-terminal of YFP (pBIF2) and PHR1(pBIF3) fused to C-terminal of YFP were used to infiltrate *N. benthamiana* leaves. After three days leaf discs were analyzed by confocal microscopy.
- C) Analysis of the cellular localization of ASK18. Five-day-old *Arabidopsis* plants expressing ASK18-GFP roots were stained with propidium iodide and analyzed in confocal microscopy (left). *N.benthamiana* was infiltrated with *Agrobacterium* carrying ASK18-GFP, after 3 days leaf discs were analyzed in confocal microscopy (right). Bars=50µm.

Therefore, we propose that ASK18 may have a role in PHR1 degradation in response to As(V). Accordingly, using transient expression of 35S:ASK18-GFP in *N. benthamiana* we found that ASK18 is localized in the nucleus as it has previously described for PHR1 (Rubio et al., 2001). This result was confirmed in a *Arabidopsis* stable transgenic line expressing ASK18-GFP (Figure 10C). In order to confirm the array data we studied the kinetic expression of *ASK18*, using qRT-PCR, in wild-type plants in response to As(V). The results show that *ASK18* is rapidly induced in response to As(V) with an inverse kinetics respect to *PHT1;1* expression (Figure 11A) or PHR1 degradation (Figure 9A). Furthermore, ASK18 was not induced by As(III), Zn or NaCl, suggesting that *ASK18* is specifically induced by As(V) (Figure 11B). All these observations prompt us to suggest that ASK18 is involved in PHR1 degradation in response to As(V). To study this possibility, we studied PHR1 stability in transgenic lines over expressing ASK18.

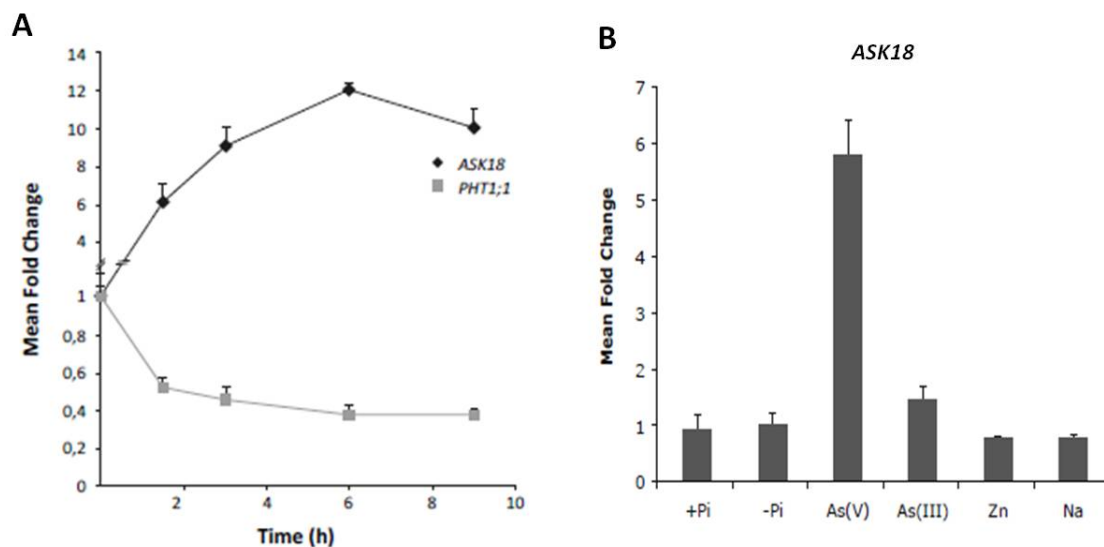


Figure 11: ASK18 is specifically induced by As(V): (A) qRT-PCR analysis of *ASK18* and *PHT1;1* expressions. Wild-type plants (Col-0) were grown in +Pi medium for 7 d, transferred to -Pi for 3 d and then to -Pi medium alone or with 30 µM As(V) for indicated timings. Values show mean \pm SD (n=3). (B) qRT-PCR analysis of *ASK18*

expression. Wild-type plants (Col-0) were grown in +Pi medium for 7 d, transferred to -Pi for 3 d and then to -Pi medium alone or with different stimulus, +Pi(1mM), As(V)(30 μ M), As(III)(30 μ M), Zn(100 μ M) or Na(200mM) for 6h. Values show mean \pm SD (n=3).

Towards this, two constructs; ASK18 fused to HA (ASK18-HA) and PHR1 fused to GFP (PHR1-GFP), both under the control of 35S promoter were transiently expressed in *N. benthamiana* leaves. Western-blot analysis using extract from these *N. benthamiana* leaves showed that PHR1 and ASK18 tagged proteins individually expressed are stable in *N. benthamiana*. However, coexpression of both proteins causes PHR1 degradation (Figure 12A). To analyse if PHR1 degradation is specifically mediated by ASK18 we expressed other ASK protein, ASK1 fused to HA. Using the same strategy as followed for ASK18 we found that ASK1 expression do not alter PHR1 stability (Figure 12B). Similar results were obtained when we expressed ASK2, another member of the ASK family (data not shown).

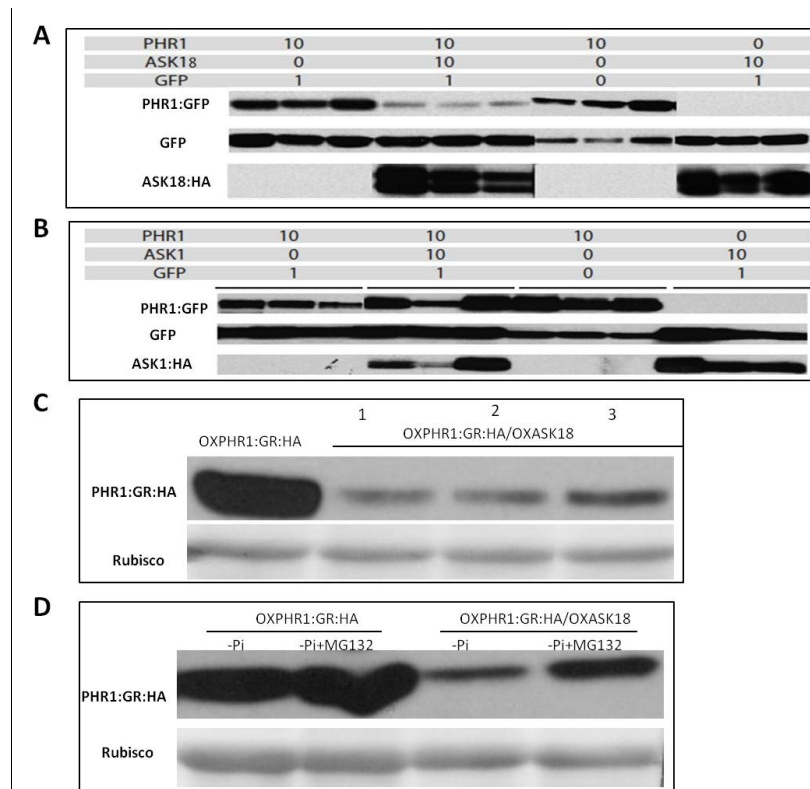


Figure 12: Western-blot analysis of PHR1 degradation.

(A)&(B) *N. benthamiana* plants were agroinfiltrated with PHR1:GFP alone or together with ASK18:HA or ASK1:HA. Samples were collected after 3 days of infiltration for analysis. 35S:GFP was used as control.

(C) *Arabidopsis* ASK18/GR:PHR1-HA and GR:PHR1-HA over-expressing lines were grown on +Pi medium for 7 d and then transferred to -Pi media supplemented with 5 μ M Dexamethasone for 3 days. Roots were collected for analysis. Three independent lines were analyzed. Rubisco was used as loading control.

(D) ASK18/GR:PHR1-HA and GR:PHR1-HA over-expressing lines were grown as described in Figure 11C, and incubated with or without 50 μ M MG132 for 8 hrs. Roots were collected for analysis.

These results supports that ASK18 specifically mediates the degradation of PHR1. To confirm this result we produced stable transgenic plants over-expressing both GR:PHR1-HA and ASK18 driven by a 35S promoter that we named OXGR:PHR1:HA/OXASK18. Western-blot analysis using three independent OXGR:PHR1:HA/OXASK18 lines, showed

that when ASK18 was coexpressed with PHR1, the latter was remarkably lower to that observed in plants expressing PHR1 alone (Figure 12C). To confirm that the PHR1 degradation observed in the transgenic lines is via proteasome we treated OXPHR1:GR:HA and OXGR:PHR1:HA/OXASK18 transgenic lines with MG132. Western-blot analysis shows that MG132 stabilized the PHR1 in OXGR:PHR1:HA/OXASK18 transgenic line (Figure 12D).

Accordingly, the Pi starvation responsive genes that are direct targets of PHR1 was compromised in OXGR:PHR1:HA/OXASK18 over-expressing line in –Pi conditions. qRT-PCR data show that the expression of genes (*PHT1;1*, *At4*, *SQD1*, *RNS1*) that are up regulated by PHR1 in response to Pi starvation were repressed in the presence of ASK18 (Figure 13A) supporting that PHR1 activity is reduced in OXGR:PHR1:HA/OXASK18 line due to the PHR1 degradation mediated by ASK18. To confirm this result we analysed the As(V)-tolerant phenotype of plants expressing both ASK18 and PHR1 compare to plants expressing PHR1 alone (Figure 13B).

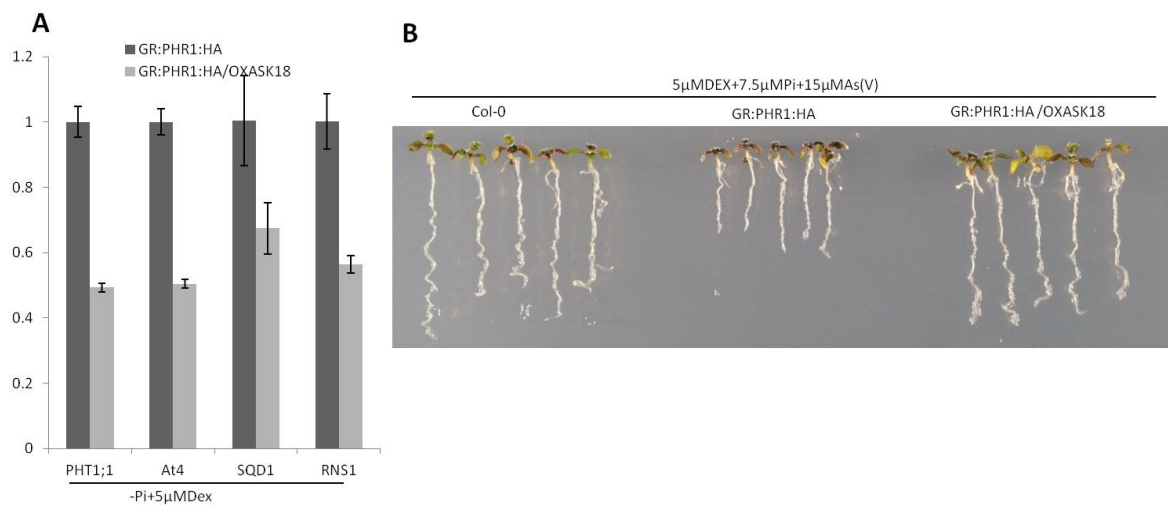


Figure 13: *Arabidopsis* ASK18/GR:PHR1-HA over-expressing line abolished molecular and physiological phenotype of GR:PHR1-HA plants. (A) qRT-PCR analysis of the expression of some PHR1 direct-target genes (*PHT1;1*, *At4*, *SQD1*, *RNS1*). *Arabidopsis* ASK18/GR:PHR1-HA and GR:PHR1-HA over-expressing lines were grown on Johnson +Pi for 7 d, and then transferred to –Pi for 3d. Values shown mean \pm SD (n=3). (B) Phenotypic analysis of wild-type plants (Col-0), PHR1 overexpressing line (GR:PHR1-HA) and ASK18/GR:PHR1-HA line in response to As(V). Plants were grown on Johnson media containing 7.5 μ M Pi and 15 μ M AsV supplemented with 5 μ M dexamethasone for 10 days.

As we expected, GR:PHR1:HA over expression provoked a sensitive phenotype to As(V) compared to that observed in wild-type plants. We found that ASK18 over-expression in GR:PHR1-HA background counteracts the As(V) sensitive phenotype

associated with PHR1 overexpression due to the PHR1 restoration to wild-type levels. We conclude that ASK18 mediates PHR1 degradation being an essential component of plant adaptation to the presence of As(V).

9. Expression of *ASK18* in response to As(V) is modulated by the detoxification machinery of the plant

PHT1;1 recovery depends on detoxification capacity of the plant (Figure 8B and 8C). In addition we showed that *PHT1;1* reactivation requires PHR1. Since ASK18 is crucial for PHR1 stability and ASK18 expression is tightly controlled by As(V), we decided to explore if the detoxification capacity of the plant alters the As(V) responsiveness of *ASK18*. We have found that induction of *ASK18* in response to As(V) is higher in BSO pre-treated as well as in *cad1-3* and *pad2-1* backgrounds compared to Col-0 (Figure 14 A&B) suggesting that the lack of PCs make *ASK18* up regulation crucial to degrade PHR1 and to keep *PHT1;1* repressed. These results tempt us to speculate that phytochelatin might act as signal molecules that modulate *ASK18* transcriptional regulation providing an integrated mechanism that adapt plant detoxification capacity to As(V) uptake rate. Further experiments will be required to explore this exciting possibility.

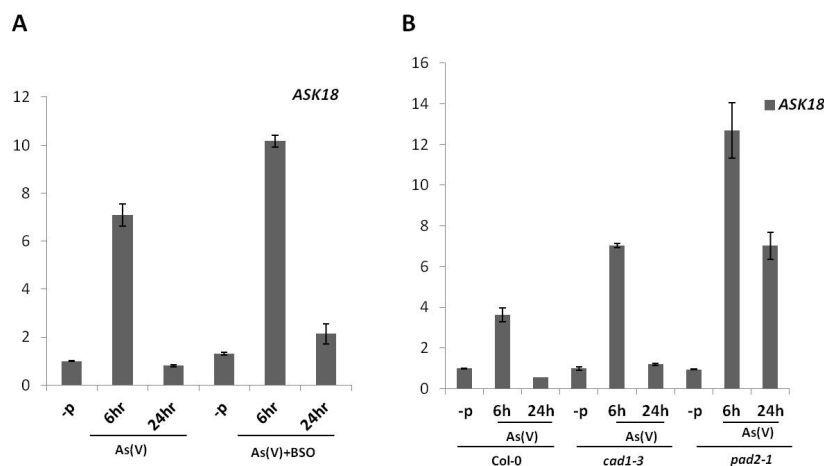


Figure 14: As(V) induced expression of *ASK18*, depends on detoxification status of the plants. (A) & (B) qRT-PCR analysis of *ASK18* expression. (A) Wild-type plants (Col-0) grown in +Pi medium for 7 d, transferred to -Pi or -Pi supplemented with 50μM BSO for 3 d and then to -Pi medium alone, with 30 μM As(V) or 30 μM As(V) and 100μM BSO for indicated timings. Values show mean ±SD (n=3). (B) Wild-type plants (Col-0), *cad1-3*, and *pad2-1*, mutants were grown in +Pi medium for 7 d, transferred to -Pi 3 d and then to -Pi medium alone or with 30 μM As (V) for indicated timings. Values show mean ±SD (n=3).

10. Role of Cytokinins in the As(V) response.

Cytokinins (CKs) have been described as a repressor of the *PHT1;1* induction in response to Pi-starvation (Franco-Zorrilla et al., 2002). In addition, CKs also act as negative regulators of sulfate transporters (SULTRs) that are essential components of phytochelatin biosynthesis (Maruyama-Nakashita et al., 2004). We therefore decide to explore what is the role of this hormone in As(V) response, particularly in the context of *PHT1;1* and *SULTR1;2* repression pattern in response to As(V). First we explore the effect of As(V) on CK signaling. Toward this, we analyzed the expression in response to A(V) of Isopentenyltransferase genes (*IPT*) and Cytokinin Dehydrogenase genes (*CKX*), that encodes enzymes relevant for CK biosynthesis and degradation, respectively. Expression analyses of 8 *IPT* genes show that only 4 of them were detected at this *Arabidopsis* developmental stage (*IPT1*, *IPT3*, *IPT5* and *IPT7*) as it was previously described (Miyawaki et al., 2006). Out of these four transcripts, three of them (*IPT3*, *IPT5* and *IPT7*) were remarkably down-regulated after As(V) exposition (Figure 15A). Similarly, the kinetic analysis of 7 *CKX* genes in response to As(V) shows that five out of six genes that showed expression (*CKX1*, *CKX3*, *CKX4*, *CKX5*, *CKX7*) were also repressed by As(V) (Figure 15B).

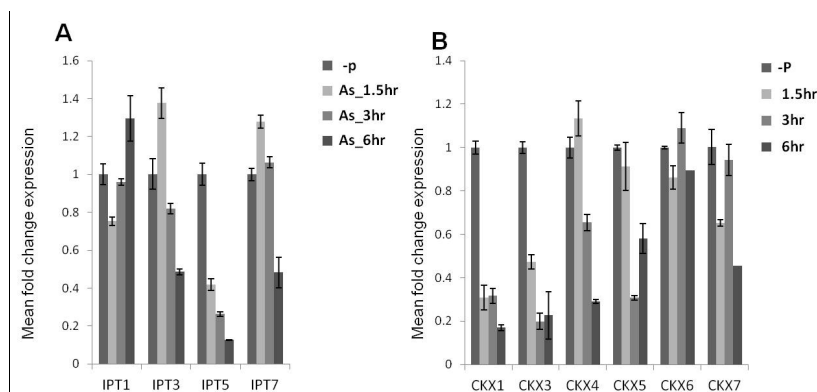


Figure 15: As(V) inhibit the expression of the genes related with cytokinin metabolism: Expression analysis of *IPT*(A) and *CKX* (B) genes by qRT-PCR. Col-0 seeds were grown on Johnson +Pi media for 7days and then transferred to Johnson -Pi for 3 days. Pi-starved plants were exposed to 30μM As(V) at different times. Values shown mean ± SD (n=3).

Furthermore, we found that in the microarray analysis of plants exposed to As(V) for 8h, CK response regulators; *ARR3*, *ARR4*, *ARR7* and *ARR9*, cytokinin receptor *CRE1/AHK4* were repressed. These results suggest that in the presence of As(V) the endogenous levels of cytokinin decreases as a result of down-regulation of genes involve in CKs synthesis and signaling.

We quantified bioactive cytokinin species in *Arabidopsis* plants exposed to As(V) for 6h and 24h and found that both tZ and its riboside form tZR, were dramatically reduced

after As(V) exposition (Figure 16). The rest of cytokinin species analyzed show a transiently reduction at 6h, followed by a recovery to initial levels. Reduction in endogenous CKs provoked by As(V) strongly suggest that this hormone act as a negative regulator of As(V) response.

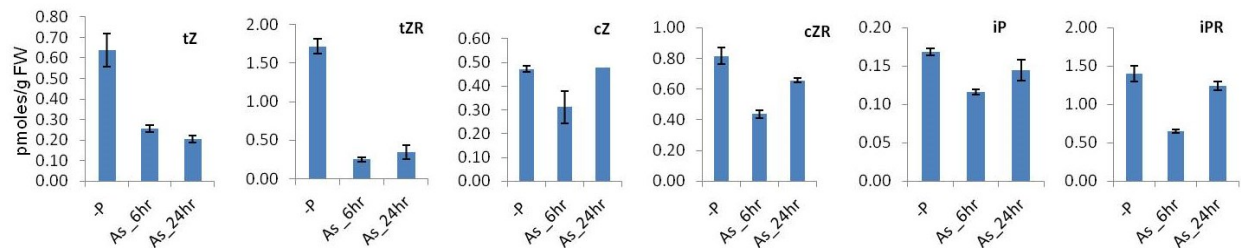


Figure 16: As(V) reduces the endogenous cytokinin content : Pi-starved plants were exposed to 30μM As(V) for 6 and 24h, respectively; after that the CK content were analyzed in whole plants. Values shown mean \pm SD (n=3).

To study the negative effect of CKs over As(V) tolerance, we used an *35S::CKX1* (Cytokinin Oxidase/Dehydrogenase1) over-expressing line. In these plants the levels of bioactive cytokinins are undetectable (Nishiyama et al 2011). *35S::CKX1* line shows a remarkable arsenate tolerant phenotype in comparison with wild-type plants (Figure. 17A and B). These analysis indicate that CKs reduction confers As(V) tolerance in *Arabidopsis*.

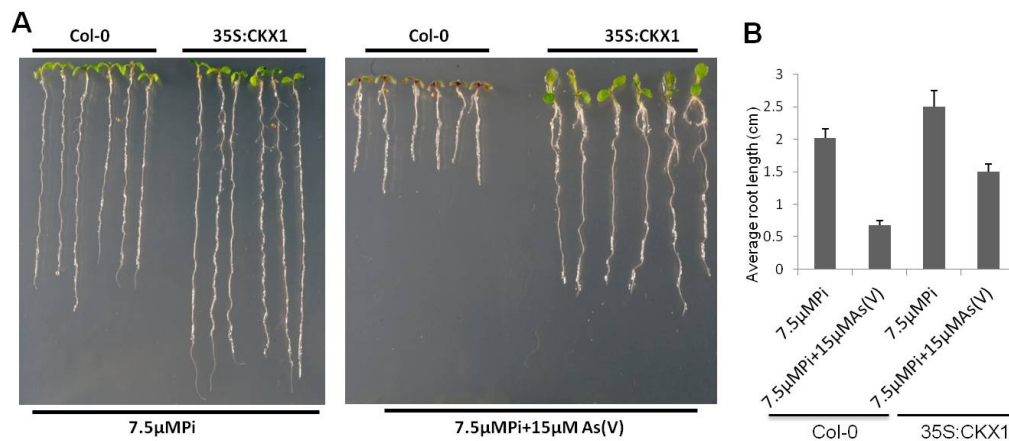


Figure 17: Phenotypic analysis of Col-0 and *35S::CKX1* in response to As(V). Col-0 and the *35S::CKX1* overexpressing line were grown on Johnson media containing 7.5μM Pi supplemented or not with 15μM As(V) for 10 days. (B) Main root length analysis. Plants from (A) were used for the measurement of main root length in the different growth condition. Values shown mean \pm SD (n=10).

10.1 The *Arabidopsis* 35S::CKX1 line exhibits high detoxification capacity

To identify the underlying molecular mechanism of As(V) tolerance in response to cytokinin depletion, we tested whether the two main mechanism of As(V) resistance in plants; transcriptional repression of the As(V)/Pi transporter *PHT1;1* and phytochelatin synthesis, are alter by cytokinin deficiency. qRT-PCR analyses shown that the rate of transcriptional inhibition of *PHT1;1* in response to As(V) has no significant difference in the 35S::CKX1 background as compared to wild type plants (Figure 18A). However, the expression of key genes involved in arsenic detoxification machinery like sulfate transporter *SULTR1;2* (Maruyama-Nakashita et al., 2004), *GSH1*, *GSH2*, *PCS1* (Sung et al., 2009) and the arsenate reductase *AtARQ1* (Sánchez-Bermejo et al., 2014) showed higher induction in the 35S::CKX1 line compared to wild-type (Figure 18B,C,D,E&F).

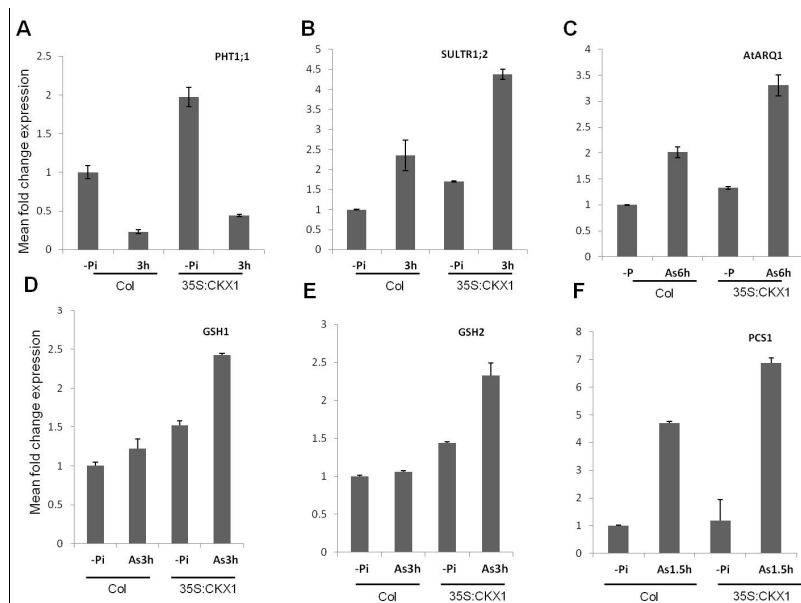


Figure 18: qRT-PCR analysis of genes related with As(V) response: *PHT1;1*, *SULTR1;2*, *AtARQ1*, *GSH1*, *GSH2* and *PCS1*. Col-0 and 35S:CKX1 plants were grown in Johnson media +Pi for 7d, transferred to -Pi for 3d and then to -Pi media alone or with 30 μ M As(V) for indicated time. The expression of these genes was analyzed in the whole seedlings except for *AtARQ1* where only roots were collected. Values show mean \pm SD (n=3).

Accordingly, microarray data of *Arabidopsis* seedlings exposed to cytokinin (Brenner et al 2005) shows that *AtARQ1* is down regulated in response to this phytohormone. The induction of these genes suggest, that As(V) tolerance exhibited by the 35S::CKX1 over-expressing line, is due to up-regulation of As(V) detoxification machinery. Indeed, 35S::CKX1 exposed to As(V) show hyperaccumulation of phytochelatin and its precursors, glutathione and cysteine (Figure 19).

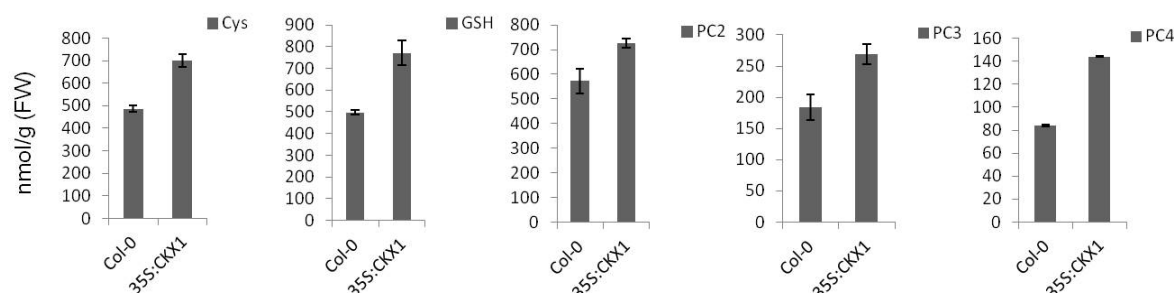


Figure 19: 35S:CKX1 line show a high detoxification capacity in response to As(V). Quantification of phytochelatin and its metabolic precursors, in Col-0 and 35S:CKX1 line. Plants were grown in Johnson media 30 μ M Pi for 7 days and then exposed to 5 μ M As(V) for 24 h. Values shown mean \pm SD (n=3).

Quantification of arsenic species shows that 35S:CKX1 plants accumulates significantly more As(III) (Figure 20) is in line with enhanced detoxification capacity.

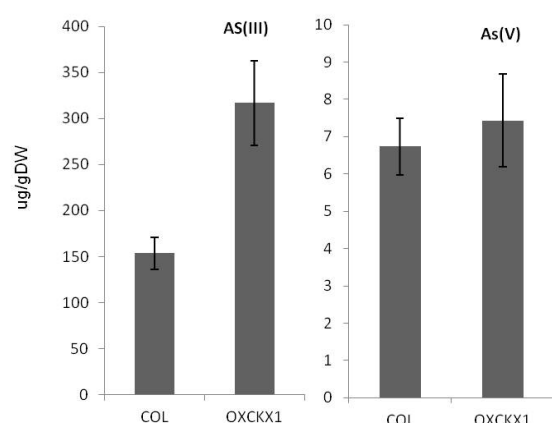


Figure 20: 35S:CKX1 accumulates more As(III) compare to Col-0. Quantification of different arsenic species, As(V) and As(III), in wild-type plants (Col-0) and CKX1 over-expressing line (35S:CKX1) exposed to 5 μ M As(V) for 24 hrs. Values shown mean \pm SD (n=3).

All of these results show that cytokinins acts as negative regulators of As(V) response. The reduction of cytokinin metabolism in response to As(V) stimulate the detoxification machinery in plants that leads to increase phytochelatin content. Further experiments will be required to understand how this phytohormone integrates the amount of phytochelatin with As(V) tolerance and whether or not this hormone mediates As(V) uptake adaptation to detoxification capacity through PHR1 stability. The identification of the cytokinin as an integral part of the As(V) response provides a novel molecular component for understanding plant adaptation to arsenate. Our results suggests that over expression of CKX1 gene may have applied biotechnological potential to increase phyto remediation capacity of plants

DISCUSSION

In response to stress, plants are able to activate its own rescue mechanism to adapt growth to the environment. Among all stresses, the presence of enormous amounts of arsenic during early earth history has been a critical threat for the inception of life and thereby crucial for plants adaptation to environmental stress early in evolution. In an oxidizing atmosphere, arsenate [As(V)] is the most abundant chemical species and due to its extreme toxicity plants must evolved fast responses once As(V) was perceived. Indeed in nature, the restriction of As(V) uptake is the first action that plants execute once the metalloid is detected (Meharg and Macnair 1990) although the molecular mechanism underlying are unknown.

Here we show that As(V) uptake is tightly regulated through the action of transcriptional repressors and activators of the Pi/As(V) transporters. The results obtained illustrate the existence of a sensitive mechanisms for As(V) perception in plants. However, Pi/As(V) similarity posses a unique dilemma for plants. When Pi is limited, phosphate transporters are induced and thereby As(V) is easily incorporated into plant cells (Raghothama 1999; Catarcha et al. 2007; Wu et al. 2011). Therefore any strategy interrupting As(V) uptake to protect plants from its toxicological effects compromise Pi acquisition and thus plant survival. To solve this delicate situation plants must have evolved fine tune mechanisms to perceive and modulate As(V) uptake. Here we found that, in response to As(V), the Pi/As(V) transporter delocalized from the plasma membrane to vacuoles by endocytosis. Along with delocalization of the transporter, As(V) also causes transcriptional repression of *PHT1;1* as we previously described (Catarcha et al 2007). The combined effect of these two mechanisms, arrest As(V) entry into plant cells. It is known that Pi and it's analogues also causes broad repression of Pi starvation responsive genes (PSR) and delocalization of the Pi-transporters that triggers its degradation (Ticconi et al., 2001; Varadarajan et al., 2002;; Bustos et al., 2010). However, in contrast to Pi, we have shown that As(V) efficiently repressed only 10% of PSR genes including Pi transporters with no marked alteration in the overall Pi starvation response. This

observation indicates that As(V) repression is a specific response that only attains for a few PSR genes. Furthermore, although both As(V) and Pi plays role in Pi transporter regulation, the rate of transcriptional repression and plasma membrane delocalization is much rapid with As(V) than with Pi that supports the existence of an As(V) specific mechanism for regulation of *PHT1;1* expression and thus As(V) perception.

1. As(V) repression of Pi/As(V) transporter is mediated by WRKY6

In this thesis we determine that the transcription factor WRKY6 regulates the repression of Pi/As(V) transporter in response to As(V). In *Arabidopsis* genome about more than 70 members of WRKY family TF are identified (Rushton et al., 2010). Previous reports have revealed that suppression of WRKY75 and WRKY45 in *Arabidopsis* resulted in reduce expression of Pi-starvation responsive genes, including the Pi/As(V) transporters (Devaiah et al., 2007; Wang et al., 2014). Here, we show that WRKY6 expression is activated in response to As(V) and participate in Pi/As(V) transporters repression during As(V) stress. WRKY transcription factors are involved in plant responses to a wide variety of environmental stresses (Rushton and Somssich, 1998; Robatzek and Somssich, 2002; Rushton et al., 2010). In fact, WRKY6 was initially implicated in the control of gene expression during plant senescence and more recently in Pi and boron starvation (Robatzek and Somssich, 2002; Chen et al., 2009; Kasajima et al., 2010). In *Arabidopsis* WRKY6 positively influences the senescence- and pathogen defense-associated PR1 promoter activity and also induces the expression of SIRK gene encoding receptor-like protein kinase during senescence (Robatzek and Somssich, 2002). WRKY6 knock out lines of *Arabidopsis* are sensitive to boron deficiency indicating its role in Boron nutrient uptake and homeostasis (Kasajima et al., 2010). In our conditions we did not observed increased As(V) sensitivity in the WRKY6 knock out line, suggesting that other members of the WRKY family may also contribute to the repression of the Pi transporters by As(V). However, *PHT1;1* expression is not repressed in response to As(V) in the *wrky6*-TDNA background, indicating incomplete functional redundancy.

Members of the WRKY family can act as activators or repressors of distinct biological functions including Pi homeostasis. In fact, WRKY6 is described as a repressor of

PHO1, which encodes a plasma membrane protein involved in Pi loading into xylem (Chen et al., 2009). The ability of WRKY6 to repress both extracellular As(V) uptake and vascular Pi loading suggests that it may operate in an integral strategy to prevent arsenic accumulation in plants. However, once As(V) is incorporated into cells, it is rapidly reduced to As(III) and is transported to the aerial part of the plant (Dhankher et al., 2006). In addition, arsenic amount in shoots of *pho1* mutant is similar to or slightly higher than that observed in wild-type shoots (Quaghebeur and Rengel, 2004), suggesting that arsenic content in the aerial part of the plant is at least partially independent of PHO1 regulation and thus of WRKY6 action. WRKY6 transcriptional repression of the Pi transporter nonetheless leads to a clear As(V) tolerance phenotype. These findings reinforce the importance of WRKY6 as an essential component in the regulation of Pi/As(V) transporters in response to As(V).

2. ASK18, an *skp1* like protein mediates degradation of PHR1 in response to As(V)

The regulation of Pi transporter expression during Pi deficiency is extensively studied (Chiou and Lin., 2011; Jain et al., 2012). During Pi starvation the Pi transporter PHT1;1 up regulation is controlled by the transcriptional activator PHR1. PHR1 is a master regulatory protein of the phosphate starvation response first isolated in the model plant *Arabidopsis* (Bustos et al., 2010; Rubio et al., 2001). In response to Pi, PHR1 activity is regulated by postranslational mechanisms (Miura et al., 2005) or interaction with a sequestering protein (SPX) in a Pi dependent manner (Puga et al., 2014; Qundan et al 2014; Wang et al., 2014). Therefore the activation or suppression of PHR1 is a key strategy to modulate the expression of the Pi-transporters in response to different Pi regimens. Although the role of SPX or post translational modification of PHR1 in the As(V) response remains to be solved, here we found that PHR1 is rapidly degraded in response to As(V). Furthermore, PHR1 degradation providing an additional mechanism to prevent PHT1;1 reactivation in addition to WRKY6 mediated repression. However PHT1;1 repression kinetic in the *phr1* mutant is identical to that observed in wild type plants suggesting that WRKY6 repressor is self-sufficient to down-regulate PHT1;1 in response to As(V).

Here we have found that PHR1 degradation is tightly coordinated with the As(V) induced ASK18 protein supporting that PHR1 stability is controlled via proteasome. In fact the presence of proteasome inhibitors prevent PHR1 degradation in response to As(V). ASK18 is an **S**-phase **k**inase-associated **P**rotein 1(skp1), the subunit of the SCF (Skp1, Cullin, F-box) E3 ubiquitin ligase complex. Genetic and molecular studies indicate that mutations in any of these core components of SCF complex can affect *Arabidopsis* growth and development that explains the importance of each component for SCF functionality (Shen et al., 2002; Yang et al., 1999). Skp1 is an adapter that links highly variable F-box protein to cullin (Figure 21) (Zhen et al., 2012).

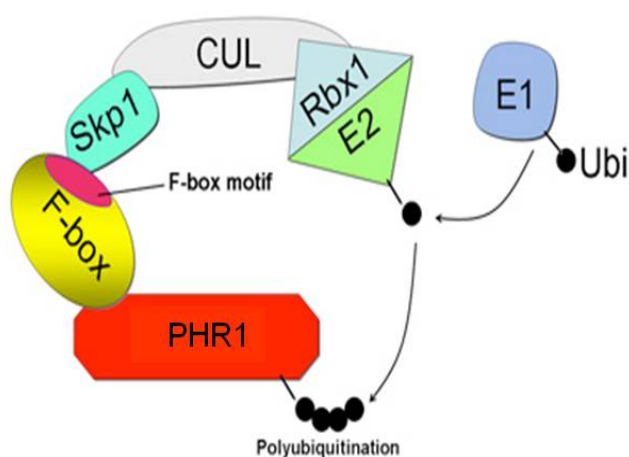


Figure 21. The structure of the SCF E3 ubiquitin ligase complex: The PHR1 protein is the target for destruction. The F-box protein(Unknown) binds PHR1 via its C-terminal domain and interacts with Skp1 (ASK18) through the N-terminal F-box motif. The Cullin (CUL1) backbone binds the ASK18 and RING-finger protein, RBX1. RBX1 binds the E2 ubiquitin conjugating enzyme, the source of ubiquitin. Ubiquitin (Ub) binds E1 (or ubiquitin activating enzyme) via a thiol ester bond to the E1. Next, the ubiquitin moiety is transferred to the E2, which binds to the SCF E3, which catalyzes transfer of Ub from E2 to the substrate PHR1. (Image modified from Ariizumi and Steber)

Among the 21 predicted skp1 homologs called ASKs (*Arabidopsis* skp1), only ASK1 and ASK2 are functionality characterized (Zhao D, et al., 2003; Kuroda H et al., 2012). It is well described that F-Boxes in SCF complexes are known to provide substrate specificity to decide which protein to degrade and when (Ho M et al., 2008). However, we have observed that ASK18 physically interacts with PHR1 *in vivo*, and ASK18 overexpressing line degrades PHR1 by abolishing the arsenic sensitive phenotype of PHR1. Our results suggest that specificity of PHR1 degradation is regulated by ASK18 by recruiting endogenous F-Boxes. PHR1 degradation is an additional mechanism to promote the suppression of Pi/As(V) transporter. This lead us to conclude that *PHT1;1* expression during As(V) response is modulated by both the action of transcriptional repressor and degradation of transcriptional activator. Further experiments using *ask18*-TDNA and the identification of

PHR1 specific F-boxes will shed light on the mechanism of ASK18 dependent degradation of PHR1.

3. Reactivation of *PHT1;1* mediated by PHR1, depends on detoxification machinery mediated by cytokinins

Our results indicates that, the reduced As(V) uptake in the over-expressing WRKY6 line leads to enhanced arsenic accumulation. Similarly, we have shown previously that an As(V) tolerant mutant *pht1;1-3* with reduced As(V) uptake also exhibits increased arsenic content (Catarcha et al., 2007). This result prompt us to speculate that As(V) uptake rate may be coordinated with arsenic detoxification capacity. One intriguing observation found here is that, the *PHT1;1* repressed transiently during the 1 to 9hrs of As(V) exposure but recovered its expression during 24 hrs even in the presence of As(V). The absence of *PHT1;1* reactivation in the *phr1* background indicates that this transcriptional activator is an essential component for *PHT1;1* recovery. Accordingly both *PHT1;1* and PHR1 kinetics are intimately coordinated in response to As(V). Furthermore, the absence of *PHT1;1* reactivation in *cad1-3* and *pad2-1* mutants altered in PCs biosynthesis supports that *PHT1;1* reactivation is regulated by the detoxification machinery of plants. It is known that arsenic induces synthesis of phytochelatins (PCs) and the genes involved in PC synthesis are arsenic responsive (Sung et al., 2009). Our results indicates that the induction of PC synthesis genes is inverse to the *PHT1;1* repression during early arsenic response which support our idea that *PHT1;1* repression allows the plant to activate the detoxification machinery to cope with arsenic. Once the cell is ready to face arsenate toxicity, the *PHT1;1* transcription will be reactivated in coordination with PCs content and As(V) uptake.

Plants exposed to mild biotic and abiotic stress conditions exhibit different kind of stress-induced morphogenic and adaptive responses including changes in their hormones profile as part of a plant general acclimation strategy, whereby growth and detoxification mechanisms are reprogrammed to reduce exposure to stress (Müller-Xiang et al., 2014; Shi et al., 2014; Iglesias et al 2014; Sharma et al., 1996; Yalpani et al., 1994). Abiotic stresses, such as drought, salt, cold significantly altered the CK metabolism in plants (Werner T et al., 2010; Nishiyama et al., 2011; Jeon et al., 2013). Indeed, Pi deficient conditions provoke a

reduction in the amount of CKs (Niu YF et al., 2013). Here we have shown that in response to arsenic, plants reduce the endogenous CKs content that leads to enhance detoxification capacity by increasing glutathione and phytochelatins as part of an acclimation strategy.

Microarray studies suggest that As(V) stress downregulate the CK metabolism genes (Fu SF et al., 2014). In good accordance with the repression of CK metabolism genes, we observed that during arsenic stress, the content of tZ type CKs were significantly reduced and iP and cZ type were transiently affected supporting that the most biologically active CK acts as negative regulators of adaptation to As(V) stress conditions. Similar results were observed in response to drought or salt stress where tZ type was decreased (Nishiyama et al., 2011). One of the possible explanation for reduction CKs in response to As(V) is the repression of *CYP735A* which is responsible for the biosynthesis of tZ-type CKs in roots (Ramireddy E et al., 2014; Kiba T et al., 2013). To investigate the biological significance of reduction in CK content during arsenic stress, we performed phenotypic analysis of arsenic tolerance in *35S:CKX1* transgenic lines that has undetectable levels of CKs (Nishiyama et al., 2011). It is known that the Pi-transporter *PHT1;1*, responsible for As(V) uptake is negatively regulated by CKs (Franco-Zorilla et al., 2002). We thus expected that, *35S:CKX1* transgenic lines might show an As(V) sensitive phenotype. Surprisingly we found that this line exhibits a remarkable As(V) tolerant phenotype. Previous studies using CK gain-and-loss-of-function mutants indicate that CKs exerts a negative role in cold, drought and salt stress responses (Tran LS et al., 2007; Werner et al., 2010; Nishiyama et al., 2011). Quantification of arsenic species performed here revealed that *35S:CKX1* plants accumulates more As(III) as compare to wild type plants. In *Arabidopsis* and tobacco, using *CKX* genes under the control of different promoters demonstrated that these transgenic lines are able to accumulate more cadmium as well other nutrient elements, which was explained by the enhanced root system in these transgenics (Werner et al., 2010). However, here we have shown that plant adaptation to the presence of As(V) involves the restriction of As(V) uptake, by suppressing the Pi/As(V) transporter (Catarchea et al., 2007) and activation of the detoxification machinery. The analysis of *PHT1;1* in *35S:CKX1* transgenic line background revealed that even though the basal level of *PHT1;1* expression

is higher compared to wild type plants, the repression rate by As(V) is similar to that observed in wild type plants indicating that As(V) restriction is CK independent. Once As(V) enters the cell the first step for arsenic detoxification is the reduction of As(V) to As(III) by the action of As(V) reductases (Rosen., 2002). The reduced As(III) is complexed with phytochelatins (PCs) and sequestered into the vacuole (Figure B) (Mendoza-Cózat et al., 2011). It is demonstrated that in arsenic hypertolerant *H. lanatus* during As(V) exposure, As(V) reduction capacity by arsenate reductases is the rate limiting step in As(V) induced PC accumulation rather than by PC synthetic capacity itself (Bleeker et al., 2006). Thus, the analysis of arsenate reductase *AtARQ1* expression in *35S:CKX1* performed here shows that *AtARQ1* responsiveness to As(V) is higher in the CKX1 overexpressing lines which agrees with the fact that arsenate reductase *AtARQ1* is down regulated by CKs (Brenner WG et al 2005).

As(III) promotes the synthesis of sulfur containing thiols (non-protein thiols; NPTs) (Sneller et al., 1999; Schmöger et al., 2000). The synthesis of NPTs requires precursors such as cysteine and GSH and thus sulfur demand for the plant growth (Nocito et al., 2006). Mayurama-Nakashita et al (2004), reported that, sulfate transporters are negatively regulated by CKs. Here we show that the sulphur transporter *SULTR1;2* is upregulated in the *35S:CKX1* background and overall is coordinated with up regulation of other genes like *GSH1*, *GSH2*, and *PCS1* that contributes for higher accumulation of glutathione (GSH) and PCs particularly PC4. It is described that longer chain PCs contribute for more efficient detoxification of metals due to higher metal binding capacity and formation of more stable complexes (Bleeker et al., 2006; Matsumoto et al., 1990). Our results are in agreement with the previous report of Srivastava et al (2009) where, they demonstrated that in *Brassica juncea* the arsenic tolerance of genotype TPM1, is due to up-regulation of sulphate transporters and down-regulation of CK-responsive genes. Our results demonstrate that, the As(V) tolerance phenotype of *35S:CKX1* is due to increased efficiency of arsenic detoxification. All these results prompt us to conclude that CK reduction content in response to arsenic provokes increased arsenic sequestration capacity and therefore the

observed arsenic tolerance phenotype exhibited by the CKX1 overexpressing lines is independent of increased root biomass.

Till date many transgenic approaches are followed to manipulate one or more genes for the phytoremediation of heavy metal contaminated environments (Zhu and Rosen., 2009). However, for efficient use of this technology it is necessary to use more complex system-wide approach that integrates all the required responses to achieve arsenic accumulation. Toward this the identification of master regulatory genes underlying the arsenic response will be crucial. Our results show that the alteration of endogenous CKs promote arsenic accumulation. However, constitutive *CKX1* expression infers pleiotropic developmental effects (Werner et al., 2003). Modulation of CKs content in a cell type specific manner may overcome this limitation and thus will permit to consider CKs manipulation as a suitable approach to enhanced phytoremediation capacity in the future.

In this work, we have observed that in *cad1-3*, and *pad2-1* mutants altered in PCs biosynthesis, the PHR1 regulator, ASK18, shows higher level of expression, suggesting that phytochelatins might acts as repressor of ASK18 (Figure 22). One fascinating possibility is that phytochelatins itself may act as signal molecules to modulate the *PHT1;1* repression/activation pattern in response to arsenic. Further experiments are required to determine if PHR1 recovery is a primary target of PCs. Our results suggest that PHR1 kinetics during arsenic exposure is controlled by ASK18 and in turn expression of ASK18 is modulated by the detoxification system of the plant. We propose that when As(V) is perceived, its uptake is restricted through *PHT1;1* repression mediated by PHR1 degradation and WRKY6 action. This rapid response accompanied by reduction in CK content allows PCs accumulation, without compromising plant survival. Once the plant is ready to cope with arsenic, As(V) uptake is reactivated in coordination with the amount of PCs. Therefore, plants display an efficient arsenic surveillance mechanism which allow plants to adapt As(V) uptake rate to the arsenic sequestration capacity.

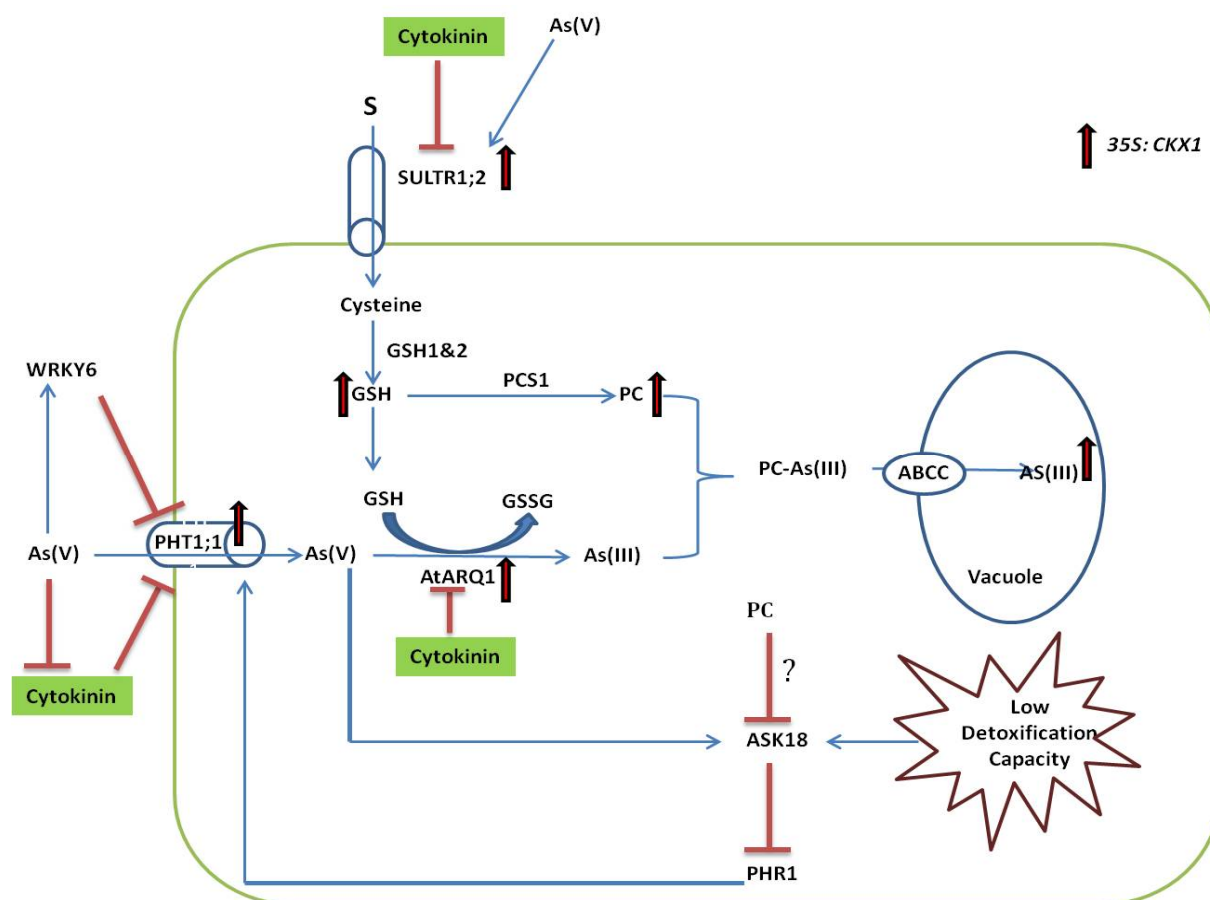


Figure 22: Model Explaining arsenic perception and detoxification mechanism.

Plants uptake As(V) through *PHT1;1*. In response to As(V), *PHT1;1* is repressed by the action of WRKY6 transcription factor and degradation of PHR1. Once the As(V) enter inside the cell it gets reduced to As(III) by arsenate reductase (*AtARQ1*) and reduced As(III) is chelated by phytochelatin (PCs) and sequestered to vacuole. When plant perceive As(V), ASK18 is induced in coordination with detoxification machinery. If the detoxification machinery is not prepared to handle incoming As(V), the up regulated ASK18 degrades the transcription factor PHR1 (Inductor of *PHT1;1*) and keep the As(V) uptake restricted by repressing *PHT1;1* until the plant detoxification machinery is ready to handle the As(V) load.

Plants also adapted modification of CK signaling pathway to survive under As(V) stress. Reduction of endogenous CKs causes the up regulation of *PHT1;1*, but at the same time WRKY6 regulates the repression of *PHT1;1*. CK also negatively regulates sulfate transporter (*SULTR1;2*) and arsenate reductase (*AtARQ1*). In the case of *35S:CKX1* the reduction of cytokinin content causes the up regulation of detoxification machinery genes which helps to handle large quantity of As(V) load by increasing the detoxification capacity of the plant.

↑ Mark indicates the components expressed more in *35S:CKX1* than Col-0.

CONCLUSIONS

1. Arsenate uptake occurs through Pi/As(V) transporters mainly by PHT1;1. During arsenic stress PHT1;1 is regulated both at transcriptional and protein level.
2. In response to As(V), PHT1;1 delocalized from the plasma membrane and As(V) uptake is restricted.
3. *PHT1;1* is transcriptionally repressed by the As(V) responsive transcription factor *WRKY6*.
4. The reactivation of *PHT1;1* requires the transcriptional activator PHR1 and is controlled by detoxification machinery of plants.
5. The transcription factor PHR1, is degraded by As(V) which is mediated by ASK18, a component of SCF E3 ubiquitin ligase.
6. The expression of *ASK18* is specifically induced by As(V) and also depends on detoxification status of the plant.
7. As(V) stress causes the reduction of endogenous cytokinin content to increase the phytochelatin concentration and thus detoxification capacity of the plants.
8. Reduction of endogenous cytokinins enhances the arsenate accumulation through phytochelatin biosynthesis.
9. Plants exhibit an efficient arsenic surveillance mechanism that provides continuous information of the arsenic and Pi status outside the plant cell that coordinates As(V) uptake rate with plant detoxification capacity.

CONCLUSIONES

1. La absorción de arsenato tiene lugar a través de transportadores de As(V)/Pi, principalmente PHT1;1. Durante situaciones de estrés por arsénico PHT1;1 es regulado tanto a nivel transcripcional como a nivel de proteína.
2. En respuesta As(V) PHT1;1 se deslocaliza de la membrana plasmática y restringiendo la incorporación de As(V).
3. *PHT1;1* es reprimido transcripcionalmente por WRKY6, un factor de transcripción de respuesta a As(V).
4. La reactivación de *PHT1;1* requiere al activador transcripcional PHR1 y es controlada por la maquinaria de detoxificación de la planta.
5. El factor de transcripción PHR1 es degradado por As(V), a través ASK18, un componente de la ubiquitina ligasa SCF E3.
6. La expresión de *ASK18* se induce específicamente por As(V), dependiendo además de estado de detoxificación de la planta.
7. El estrés por As(V) causa la reducción del contenido endógeno de citoquininas, como consecuencia aumenta la concentración de fitoquelatinas y por tanto la capacidad de detoxificación de las plantas.
8. La reducción de citoquininas endógenas aumenta la acumulación de arsenato mediante la regulación de la biosíntesis de fitoquelatinas.
9. Las plantas poseen un eficiente mecanismo de control del arsénico que proporciona información continua sobre los niveles de arsénico y Pi fuera de la célula, coordinando la tasa de absorción de As(V) con la capacidad de detoxificación de la planta.

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